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*No. 1. SIMON FLEXNER, J. W. JOBLING, and MAUD L. MENTEN. Experimental studies on tumor. Plates 1–28. (Issued June 30, 1910.) Price, $1.00.

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THE CATAPHORETIC VELOCITY OF MAMMALIAN RED BLOOD CELLS.

By HAROLD A. ABRAMSON.

(From the Laboratory of Research Medicine, Medical Clinic, the Johns Hopkins University, Baltimore.)

(Accepted for publication, March 20, 1929.)

INTRODUCTION.

The cataphoretic velocity of the red blood cells of different mammals is determined by certain surface characteristics of the cells and hence is of value in studying the constitution of the cell surface. The correlation of these values with the zoological classification of the animals may have phylogenetic or ontogenetic significance. The cataphoretic velocities of the red blood cells of a group of mammals is the subject of this communication.

HISTORICAL.

There have been a great many experiments dealing with the sign of charge of red cells and the order of speed with which the red cells of different mammals move in an electric field. These experiments, however, must be considered for the most part as qualitative, and when fine distinctions are to be drawn, very frequently erroneous. Kozawa (1) noted that the red cells of different animals could be brought to an isoelectric point at a pH characteristic for each animal, the pH decreasing in the order

Rabbit > Guinea pig > Cat, Human > Dog > Pig.

Less acid was required if the suspensions were allowed to stand. It was evident to Kozawa that some change with time took place in the suspensions which altered the surface of the red cells. Kosaka and Seki (2) have also attempted to compare the red cells of different animals. In 0.9 per cent NaCl (the pH is not given) they found the qualitative series, (order of increasing velocity)

Rabbit < Pig < Guinea pig < Man < Mouse < Rat, Cat, Dog.

If the suspending fluid was 9.5 per cent saccharose instead of saline the order just cited was reversed to

Cat, Dog < Man < Rat < Guinea pig < Pig < Rabbit.

Here again there is no mention of degree of acidity. Kosaka and Seki state further that the red cells of the rabbit in serum move to the cathode. Such data show that there probably are differences, but they cannot be used quantitatively. Kozawa's observations, namely, that the surfaces of red cells in acid media undergo an important change easily perceptible by the method of cataphoresis were confirmed and extended by Eggerth (3) and also by Netter (4). Eggerth also found that human cells at pH 5.2 and below, particularly in buffered sugar solutions, show a marked change in velocity on standing. Netter found the same for ox and horse red cells. This change occurred whenever cells remained for some time in a solution of low salt concentration. It is evident that experiments which deal with differences in red cells of different animals in acid solutions are not easily interpreted because the acid produces an unstable system at the red cell surface. One might, perhaps, speak of a "dying red cell" with death or "equilibrium" at the end of 2 hours immersion—the time at which Netter considered "equilibrium" to be present. One may properly compare red cells of different animals in a given medium only if there is no change with time. Netter found that horse red cells in serum and in buffered saline solutions moved slightly faster than ox cells in the same medium. Bernardi (5) has found the following series:

Rabbit < Cat < Guinea pig < Man < Pig.

Much of the data cited above have been obtained under uncertain experimental conditions and with practically complete disagreement amongst the various authors.

Freundlich and Abramson (6) have found that red and white cell velocity in serum did not vary very much in 10 different adult horses, although differences between red cell and white cell velocity were marked. Abramson (7) has published similar data for red and white cells in plasma of 7 different horses.

METHODS.

The method of measurement and of calculation of absolute cataphoretic velocity was performed in the manner described by Abramson (8). The apparatus employed was a modification of that first published by Northrop and Kunitz (9) and Kunitz (10). A single apparatus was used throughout. The values of mobility for this cell corresponded within 9 per cent of values determined by means of a cemented cell of the same general construction, but of different dimensions. Mobility values found by Abramson (11) for quartz particles covered by albumin in this cemented cell have been checked by the streaming potential measurements of Briggs (12) on similar surfaces of quartz membranes covered with the same protein. The velocities here given for red cells, having been checked directly and indirectly, should represent absolute mobilities certainly within 10 per cent. It will later appear that smaller differences may, on occasion, be extremely simply estimated.

The blood cell suspensions were made up as follows. One drop of freshly drawn blood (in the capillary portion of a white cell pipette) was added to 25 to 50 cc. of M/15 phosphate buffer of pH = 7.35 ± 0.03 so that an exceedingly dilute suspension of blood cells was obtained. As will appear later, the volume of the blood added, within reasonable limits, does not interfere with the constancy of cataphoretic velocity of the blood cells. The buffer employed had a specific resistance of 126 ± 1 Ohms at 25°C. Measurements of cataphoresis were made at room temperature, which usually varied between 22° and 27°C. The values in the tables are corrected to 25° by assuming that the temperature coefficient of the cataphoresis of the red cell in the medium employed is 0.02 per degree Centigrade.

The type of cataphoresis cell used is readily cleaned with cleaning mixture between measurements. This precaution was not taken, however, as experiment proved it to be unnecessary. Running tap water was drawn between measurements for 2 minutes through the cell by means of a suction pump. If it be desired to study the effect of short periods of time on mobility the cell can be turned upside down, and the particles resuspended.

EXPERIMENTAL.

The data discussed in the historical section were obtained for the most part under conditions which did not lead to a steady state without necessitating a destruction of the red cell structure. Before the cells of different animals could be compared, therefore, it was essential that a medium be employed which had no destructive effect on the cell. M/15 phosphate buffer of pH 7.4 was chosen. Table I gives the effect of time of standing on mobility. It is evident that for a large variety

TABLE I.

The Effect of Standing Time on Velocity.

There is no significant change under the experimental conditions.

Animals	Velocity at first	Velocity after standing
	μ per sec. per volt per cm.	*μ per sec. per volt per cm.*
Man 1	1.21	1.12 (12 hrs.)
" 2	1.22	1.22 (48 hrs.)
" 3	1.23	1.20 (18 hrs.)
" 4	1.30	1.29 (18 hrs.)
" 5	1.20	1.15 (24 hrs.)*
" 6	1.28	1.18 (24 hrs.)*
Opossum	1.05	1.11 (24 hrs.)
Dog	See Table III	1.55 (12 hrs.)
Cat	See Table III	1.39 (12 hrs.)
Sloth	0.97	1.00 (24 hrs.)
White rat	See Table III	1.43 (24 hrs.)

* Sickle cell anemia.

of animals (man, dog, cat, sloth, opossum, rat) the cataphoretic velocity remains unchanged in this medium for at least 24 hours. A second matter to be considered was the effect of further dilution and of repeated washing of the red cell suspensions. The suspensions studied, as Table II demonstrates, were as dilute as necessary to obtain constant values. Washing with large volumes of buffer produced no significant change in the velocities of rabbit and human cells. It is to be noted that almost the same constant value was obtained if washed cells were hemolyzed by diluted hypotonic buffer and cells subsequently added to the hemolytic mixture (which had been again made isotonic). This confirms a similar observation made by Eg-

gerth: that red cells adsorb little if any hemoglobin in media at this reaction.

The Cataphoretic Velocity of Normal Adult Human Cells.

A series of 10 normal white adults gave a mean velocity of 1.31 ± 0.02 μ per second per volt per centimeter. Both sexes were about equally represented and the ages varied from 19 to 53 years.[1] The mean of 10 normal adult Negroes velocity was 1.30 ± 0.05μ per second per volt

TABLE II.

10 cc. of a dilute suspension of red cells whose volume per cent was less than 2 per cent were washed at least 5 times with 10 cc. of N/15, pH 7.4 phosphate buffer. The residual volume was about 0.3 cc. The data are not corrected to 25°C. There is no easily perceptible change.

Exp.	Animals	Velocity before washing	Velocity after washing	Remarks
		μ per sec. per volt per cm.	μ per sec. per volt per cm.	
1	Man	1.14	1.20	
2	Man	1.27	1.29	
3	Man	1.31	1.31	Hypertension Nephritis Secondary anemia
4	Rabbit	0.50	0.52	
5	Human cells washed as above, then hemolyzed; similarly washed cells then added. See text	See table for limits of normal	1.20	
6			1.34	

per centimeter. Three members of the Yellow race possessed similar velocities in comparable experiments. This is indirectly confirmatory of the observations of Schroeder* who noted that there were no differences in speed amongst red cells of the various Landsteiner blood groups (Table III). While it is relatively simple to get values corresponding within 3 to 5 per cent on the same day, errors of meas-

* Schroeder, *Pflug. Arch. f. d. ges. Physiol.*, 1926, ccxv, 32.

[1] Another series of 10 white adults in a buffer having a slightly greater salt content gave a value of 1.22 ± 0.02 per second per volt per centimeter.

urement from day to day lead to wider variations as is evident from Table IV where the measurements were made during several weeks

TABLE III.

The Cataphoretic Velocity of Mammalian Red Cells at pH 7.35 in M/15 Phosphate Buffer.

The velocity of a human control is given only when small differences are to be considered.

Order	Animal	Number of animals investigated	Mn. observed velocities	Average deviation	Velocity of human control	Remarks
			μ per sec. per volt per cm.		*μ per sec. per volt per cm.*	
Primate	Man (white)	10	1.31	±0.02		
	Man (negro)	10	1.30	±0.05		
	Monkey (*Macacus rhesus*)	4	1.25	±0.02	1.23	2 young monkeys
Carnivor	Dog	3	1.68	±0.03		Fairly rapid hemolysis
	Cat	3	1.40	±0.01	1.32	Fairly rapid hemolysis
Ungulate	Pig	5	0.98	±0.03		Rapid spontaneous hemolysis
Rodent	Rabbit	5	0.55	±0.05		Included 2 young rabbits
	Guinea pig	6	1.11	±0.02		
	Mouse	5	1.35	±0.06	1.26	4 white mice
	Rat	5	1.45	±0.02	1.31	4 white rats
Edentate	Sloth	1	0.97			Two-toed sloth One series of measurements
Marsupial	Opossum	1	1.07	±0.02*		Adult, male

* Two series of measurements.

on a single individual. Although the mean is practically the same within the limits of error, it is evident that for fine differences in velocity between different animals a comparison must be made with a

control under as similar experimental conditions as possible. The ideal arrangement would be to study cells from different animals simultaneously in the same suspension. As will appear shortly, this is possible.

Normal White Infants.—Table V demonstrates that the cataphoretic velocity of the red cell in the new-born white infant is similar to that in the adult. During the extra-uterine life of the white race, therefore,

TABLE IV.

Measurements Repeated over 7 Weeks Showing Variation in Control.

Date	Velocity
	μ per sec. per volt per cm.
Oct. 18	1.29
23	1.26
24	1.22
26	1.30
28	1.21
30	1.19
Nov. 2	1.25
6	1.31
7	1.37
11	1.28
12	1.37
16	1.29
17	1.29
21	1.26
26	1.35

Mn = 1.28 ± 0.04.

the surface of the red cell remains, on the whole, the same. It is evident that the problem may be extended to the study of changes during intra-uterine development.

Mammals.—The data given in Table III clearly show that very wide differences in the cataphoretic velocity of the red cells of different mammals exist. The absence of an effect of age in humans has been noted. Cells of young monkeys and young rabbits had the same speed as the adults. The mean value of 0.55 per second per volt per centimeter found for the rabbit is but one-third of the speed of dog cells. In the series here presented the following order is found.

Rabbit < Sloth, Pig < Opossum, Guinea pig < Man, Monkey < Mouse, Cat,
(0.55) (0.97) (0.98) (1.07) (1.11) (1.31) (1.33) (1.40) (1.39)
Rat < Dog
(1.45) (1.65)

The number under each type of animal is the value in μ per second per volt per centimeter taken from the tables but corrected for the value of the controls when small differences are present. Thus for monkey the speed found was 1.25 per second. The control (human) was 1.23 per second. Taking 1.31 per second as the standard speed, the speed for the monkey becomes 1.33μ per second.

TABLE V.

Normal White Infants.

Baby	Age	Velocity
		μ per sec. per volt per cm.
Series I:		
No. 1	9 days	1.28
Control adult		1.22
Series II:		
No. 2	5 days	1.23
No. 3	3 hrs.	1.18
No. 4	2 days	1.23
No. 5	1 hr.	1.18
No. 6	1 day	1.27
No. 7	1 day	1.22
Control adult		1.21
Mn. infants (Series II)		1.25

It is to be emphasized that these values are for the particular experimental conditions described above. It is known that changes in pH or of salt concentration or valence can produce changes in the cells of a single type of animal greater than the variations described here for the cells of different animals.

This series has been confirmed by comparing red cells of different animals in the same medium at the same time. If rabbit cells are mixed with human cells, it is quite striking to see the human cells, which move more than twice as fast as those of the rabbit overtake

and pass the rabbit cells. In this manner it can be clearly demonstrated that mouse cells, for example, move faster than human cells. The smaller mouse cells may be easily distinguished from human cells and the greater speed of the former easily perceived. The presence of high concentration of electrolytes makes measurements less precise than in more dilute solutions because of the low particle speed and because of the increased electroendosmotic flow, with turbulence more readily occurring. The differences between mouse and human cells which have been measured in successive measurements are at the limits of the experimental error. It was consequently observed with satisfaction that these slight differences which had been measured could be confirmed by direct simultaneous observation. The fact that small amounts of heterologous plasma does not change the red cell surface to an appreciable extent under these conditions is in keeping with the previous observation that red cells can migrate through gelatin serum sols and gels without adsorbing appreciable amounts of protein (13). These experiments can be confirmed by other striking experiments which demonstrate that the red cell preserves its surface integrity. Human red cells were added to a suspension of rabbit cells in rabbit blood (diluted 1:4 with isotonic glucose). In this mixture, which contained about 2 per cent of rabbit plasma proteins, human cells moved about twice as fast as the rabbit cells. And similarly, in the converse experiment, rabbit cells in human serum (diluted 1:6 with isotonic glucose) migrated with about one-half the speed of human cells. This fact may be of use in determining the fate of intravenously injected heterologous red cells. A simple experiment (Table VI) demonstrates that while cholesterol and quartz adsorb gelatin from dilute solution in M/15 phosphate buffer at pH = 7.35, the red cell even after 24 hours has not changed its cataphoretic velocity. The red cell surface is, therefore, here also practically unaffected by the proteins present.

These experiments do not demonstrate that union of red cell with heterologous serum protein does *not* take place at all. It seems likely, in view of preliminary experiments dealing with this point, that some combination does take place in high concentrations of serum with certain components of normal inactivated serum. Whether or not the reaction is similar to that which is supposed to accompany sensi-

tization is under investigation and will be described in a future communication.

Kosaka and Seki have noted that suspending the cells in isotonic sugar solutions reverses the order of magnitude of mobility. Table VII gives data in disagreement with these findings. The velocity

TABLE VI.

The Effect of Gelatin on the Cataphoretic Velocity of Quartz, Cholesterol and Red Blood Cells in pH 7.35 M/15 Phosphate Buffer.

Particle	Cataphoretic velocity	
	Without gelatin	With gelatin
	μ per sec. per volt per cm.	*μ per sec. per volt per cm.*
Human red cell	−1.31	−1.27 (after 24 hrs. 1.23)
Quartz	−3.55	−0.39 approximate
Cholesterol	−2.25	−0.35 approximate

TABLE VII.

Red Cells Suspended in Isotonic Dextrose Solution and Dilute Phosphate Buffer (pH = 6.8).

Each value is the mean of two determinations on two animals.

Animal	Velocity	Remarks
	μ per sec. per volt per cm.	
Rabbit	1.27	
Pig	2.09	Marked hemolysis
Man	2.33	
Mouse	2.60	

order Rabbit < Pig < Man < Mouse is, as in pure phosphate buffer, also to be found in suspensions of the red cells of these animals in phosphate buffered isotonic sugar solutions.

There is no relationship between our series and the ease with which hemolysis occurs spontaneously. Pig cells are most fragile in M/15 phosphate buffer. Human and rabbit cells hardly hemolyzed at all. Cat and dog cells show a marked tendency to hemolyze.

There was no agglutination within 24 hours; nor did slow centrifugation produce agglutination.

TABLE VIII.

Patient	Age	Race	Sex	Clinical diagnosis	Velocity
					μ per sec. per volt per cm.
1 M.	19	W	F	9 mos. pregnancy	1.35
2 H.	19	W	F	9 mos. pregnancy	1.31
3 P.	34	W	F	9 mos. pregnancy	1.31
4 C.	19	W	F	9 mos. pregnancy	1.30
5 Mc.	26	W	F	Secondary anemia	1.34
6 J.	42	W	M	"Cured" (liver diet) Pernicious anemia	1.30
7 A.	32	B	F	Severe secondary anemia	1.12 (control 1.25)
8 M.	52	W	M	Nephritis; hypertension mild secondary anemia	1.34
9 P.	52	B	F	Carcinoma of colon; mild secondary anemia	1.17 (control 1.26)
10 S.	7	B	M	Sickle cell anemia	1.28
11 J.	8	B	F	Sickle cell anemia	1.20
12	3	B	M	Sickle cell anemia	1.28
13 H.	58	W	M	Pernicious anemia	1.20 (cells of different shape have a same speed)
14 F.	30	W	M	Severe secondary anemia (6 transfusions during 10 wks. previous to admission)	1.25
15 M.	20	W	F	Severe $HgCl_2$ poisoning. Very severe anemia (citrate transfusion previous day)	1.24
16 M.	45	W	F	Pernicious anemia carcinoma. Very severe anemia	1.35 (transfusion day before. Normal cells in blood suspension)

The approximate ζ-potential may be obtained by multiplying the values given in the table by the factor 13. The rabbit cells, the lowest of the series, have a ζ-potential of about 7 millivolts, while the dog cells

ζ-potential is about 21 millivolts. (Assuming dielectric constant = 80, viscosity = 0.01 for M/15 phosphate buffer at pH = 7.35).

The Red Cell in Pregnancy.

In four cases of pregnancy at term, the cataphoretic velocity of the red cells was normal. This suggests that red cells of pregnant women suspended in citrate (as in the sedimentation test) would have the same mobility as normal women, and that there is here no direct relationship between cataphoretic velocity and sedimentation velocity. This viewpoint is well borne out by related data of Netter. Netter found that horse red cells migrate very slightly faster than ox cells. Horse cells clump quickly and settle out almost at once. Ox cells on the other hand are noted for their stability in suspension without clumping. There is here evidently no relationship between low values of mobility and aggregation. The experiments of Northrop and Freund (14) and of Oliver and Barnard (15) should be consulted in this connection.

The Diseases of the Blood.—A short series of primary and secondary anemias were studied. In primary and secondary anemias, with the exception perhaps of Patient 7 A., no significant change in cataphoretic mobility has been observed. Three cases of sickle cell anemia had practically normal velocities. It is very striking to see all the cells, in anemias where extreme variations in size, shape and hemoglobin content of the cells exist, migrate with practically the same speed. Patient 16 M. had a transfusion the day previous to the examination. There were many normal, probably transfused, red cells to be seen moving with the same velocity as the distinctly abnormal cells. These observations also again demonstrate the obstinacy with which the red cell maintains the general integrity of its surface in spite of most varied circumstances.

DISCUSSION.

The recent experiments of Abramson (13), (16) in collaboration with Freundlich and with Michaelis have made it clear that neither the size nor the shape of microscopic particles suspended in electrolyte-containing media influences the cataphoretic velocity. The differences in red cell velocity given in the preceding statement are, there-

fore, almost certainly representative of the surface constituents of the different red cells. With this in mind the data presented may be discussed.

It is well known that the chemical make-up of the surface of the microscopic particle influences its cataphoretic velocity. And it is of importance to be able to ascribe the variations in velocity found amongst mammalian red cells to a correlated series of changes in the structure of the surface layers. It is more or less customary to consider the red cell surface as hydrophobic. The data of the Mudds is most pertinent and interesting (17). Yet Netter has proposed that the surface of red cells of the horse and the ox, for example, may be composed chiefly of protein. He concluded from cataphoretic experiments that the former was made up primarily of globulin while the latter was chiefly albumin. It is difficult to evaluate Netter's conclusions for his data were primarily obtained in acid solutions where, we have seen, secondary changes always occur. The large differences in mobility found amongst the various mammals, the speed of the rabbit cells of the order 0.55μ per second per volt per centimeter at the one extreme and the dog cells 1.65μ per second per volt per centimeter, at the other extreme, indicate that it is probably not strictly permissible to speak of a characteristic red cell surface in general. The speed of the rabbit cells is very close indeed to the order of magnitude of the speeds found for inert particles covered with protein. The dog cells, on the other hand, are very near the speed of 2.2μ per second per volt per centimeter given here for cholesterol. It is not intended to suggest that cholesterol is a constituent of the red cell surface but rather that substances of a somewhat similar constitution plus protein could account for the high mobility of the dog cell, with mixtures of protein plus this substance or substances producing the intermediate velocities. It may also be mentioned that there is scarcely any quantitative data on the absolute mobility of the plasma proteins and the common lipids.

Grouping the animals used according to order shows clearly that zoological Order and cataphoretic velocity do not invariably go hand in hand. Thus for the Rodents, the rabbit, guinea pig and mouse differ widely, the mouse cells having velocity values much closer to those of the Primates and Carnivora. The most striking observation

is the slow speed in the case of the rabbit cells compared with any other of the animals investigated.

The human red cell when examined in a suitable medium will probably be an excellent particle for the checking of the calibration of cataphoresis cells.

SUMMARY AND CONCLUSIONS.

1. No significant change with time (to 24 hours) in the cataphoretic velocity of certain mammalian red cells occurs when the cells are suspended in M/15 phosphate buffer at pH = 7.35. Neither successive washings nor standing effect a change.

2. In M/15 phosphate buffer at pH 7.35 ± 0.03 the following order of red cell velocity has been obtained. The numbers in parenthesis are μ per second per volt per centimeter.

Rabbit	<	Sloth, Pig	<	Opossum, Guinea pig	<	Man, Monkey	<	Mouse, Cat,
(0.55)		(0.97) (0.98)		(1.07) (1.11)		(1.31) (1.33)		(1.40) (1.39)

Rat < Dog
(1.45) (1.65)

The order, though not the absolute values, was the same in buffered isotonic dextrose. Human and rabbit cells showed similar differences when both were studied simultaneously in the serum of either. Under these conditions, there is no apparent relationship between zoological Order and cataphoretic velocity.

3. Cholesterol and quartz adsorb gelatin from dilute solution in the phosphate buffer. Red cells, on the other hand, even after 24 hours contact with gelatin solution, retain their previous velocity.

4. Pregnant and non-pregnant white female humans have the same red cell cataphoretic velocity. (The cells were not agglutinated.)

5. In a series of severe anemias no significant change in cataphoretic velocity was in general apparent, although marked changes in the morphology of the red cells were present.

I am indebted to Professor L. Michaelis for much valuable advice received in connection with this investigation.

Through the kindness and cooperation of Professors Hartman, Richter, Longcope and of Dr. M. Smith much of the material investigated was made accessible.

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THE VISUAL ACUITY OF THE HONEY BEE.

By SELIG HECHT and ERNST WOLF.*

(From the Laboratory of Biophysics, Columbia University, New York.)

(Accepted for publication, March 26, 1929.)

I.

Nature of Work.

1. The visual acuity of the human eye varies in a specific way with the illumination. At low intensities visual acuity is low; as the intensity increases, visual acuity increases with it, at first slowly, then more rapidly; and finally at very high illuminations further increase in intensity produces no augmentation of acuity (Uhthoff, 1890; Koenig, 1897).

This extraordinary dependence of visual acuity on illumination has been known for many years. However, it has remained without explanation until we recently suggested a theory to account for it (Hecht, 1927–28). The idea underlying this explanation is as follows. Visual acuity measures the resolving power of the retina. The resolving power of a surface composed of discrete receiving elements varies inversely with the distance between the centers of the elements; or more generally, it varies with the number of elements present in a unit area. The retina is such a surface since it is made up of discrete rods and cones. The variations in visual acuity with illumination would therefore suppose that the number of visual elements per unit area of retina varies with the intensity. But since the number of rods and cones in the retina is fixed anatomically one must suppose it to vary functionally. To accomplish this, it has been assumed that the thresholds of the retinal elements are not the same, but are distributed in relation to the illumination in the usual manner of populations. Consequently the number of elements functional in a given area varies with the illumination; and when expressed quantitatively,

* Fellow, International Education Board.

this relation describes in detail the dependence of visual acuity on the intensity of illumination.

This work on visual acuity is confined to the eye of man. Its theoretical implications are many (Hecht, 1927–28) and involve consideration of the problems of intensity perception and intensity discrimination; it may even concern the matter of color reception and hue discrimination. The ideas underlying it should therefore be tested in a variety of ways. If the explanation of the dependence of visual acuity on illumination which we have proposed is generally valid; in other words if the number of receptor elements functional in a sense organ does vary with the intensity of the stimulating agent, then visual acuity should vary with the illumination in other animals in a manner resembling that in man.

There exist, however, no measurements of this nature with any other organisms. We have therefore studied the visual acuity of the arthropod eye, a sense organ which is built and is functional on a wholly different principle from the vertebrate eye (Exner, 1891).

2. The problem of studying the vision of animals other than man is made difficult by the impossibility of verbal communication between the animal and the experimenter. The best method of solving this difficulty up to the present has been by means of the conditioned reflex, used originally by Pavlov (1927) with mammals, and in a modified form by v. Frisch (1915) with bees. Though we have nothing but praise for this method and for many of the results achieved by it, we nevertheless tried to find a more direct one, which would involve no training of animals.

We started with the common observation that most animals with eyes respond to a sudden movement in their visual field, and in terms of the following considerations converted it into a method of measuring visual acuity quantitatively. If the visual field of a sensitive animal is made up of a pattern of dark and illuminated bars of equal size, the animal will respond to a displacement of this field only when it can distinguish the components of the pattern. In case the animal cannot resolve the black and white bars, the field will appear uniformly illuminated and displacement of the pattern will elicit no response. If visual acuity varies with illumination, then the capacity to respond to these movements in the visual field will depend on the illumination

and on the size of the pattern. One could in this way determine the relation between the size of the bars in the pattern and the minimum illumination at which it causes a response in the animal.

3. Our experiments were made with the honey bee because of its abundance and its genetic uniformity. It is sensitive to changes in its visual field, and responds by a reflex, sidewise movement of the head and thorax. If the bee is crawling, the response becomes evident by a sudden change in the direction of its progression, which is opposite in sign to the movement in its environment.

We prepared a series of plates composed of equally wide opaque and translucent bars, each plate having a different size of bar. Our experiments then consisted in determining for each size of pattern the minimum illumination at which a bee will just respond to a movement of that pattern. The reciprocal of the visual angle subtended by each size of bar is then the visual acuity of the eye at the corresponding illumination. The results show that the visual acuity in the honey bee varies with the illumination in a way which is similar to the human eye.

II.

Apparatus and Procedure.

1. The details of the apparatus which we used in these experiments can be made out with the help of Fig. 1. A source of light (1000 watt, concentrated-filament, mazda lamp) in dark room *B* illuminates a plate of opal glass in the wall of an adjacent dark room *A*, in which the experiments are performed. The opal plate is immediately behind an accurately controlled diaphragm (Fig. 1) the opening of which determines the area of the plate which radiates light into dark room *A*. This light is reflected upward by a mirror, and impinges on a piece of opal glass 25 x 15 cm. in size. Immediately upon this opal glass plate rests a piece of transparent glass of the same size, with the experimental opaque and transparent bars on it. These two pieces of glass,—the opal plate and the pattern plate,—fit into the opening in a frame which is moved by an attached knob, and which slides freely in a pair of grooves in a larger frame. The movement of the frame with the two plates constitutes the moving visual pattern.

The bee is confined in a glass compartment 10 x 10 cm. and 3 cm. high, held about a centimeter above the pattern. The bottom of the compartment is the plate on which the bee crawls. It is an ordinary photographic plate with the halide completely removed but the gelatine left so as to serve as a foothold for the crawling bee. These plates are used because they are uniform; they may be removed very frequently and washed and dried.

The bee's creeping compartment and the movable black and white stripes of the visual field are tilted at an angle of about 30°. Because of the bee's negative geotropism this tends to make the animal crawl upward in a straight line, so that

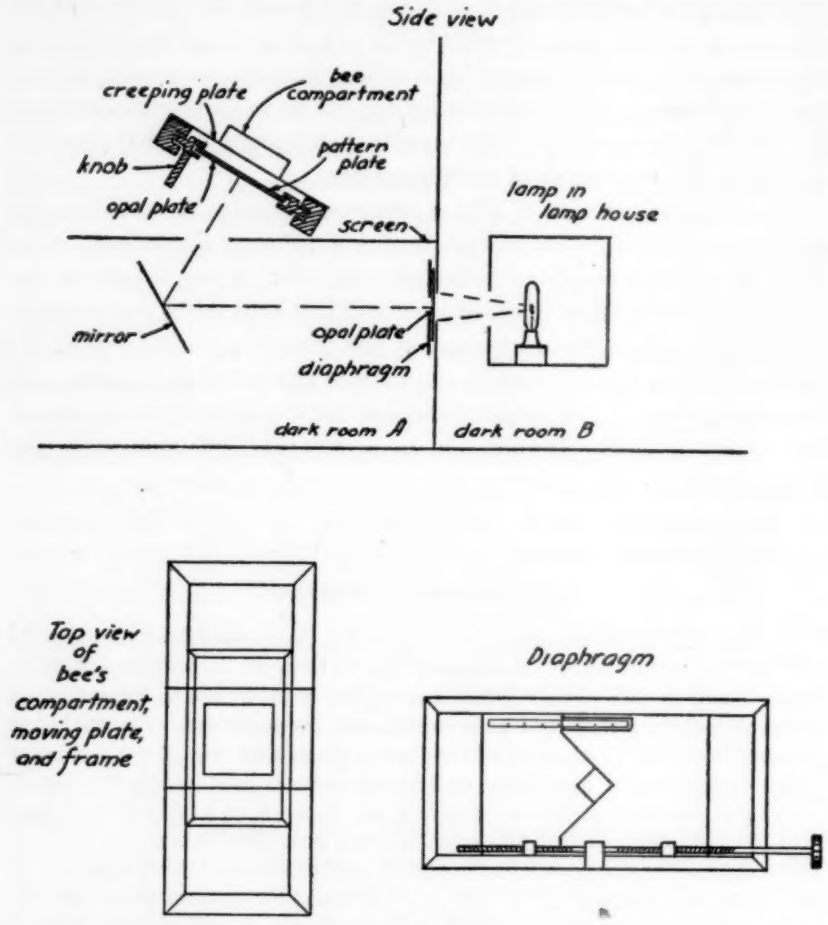

FIG. 1. Diagrammatic representation of apparatus for measuring the visual acuity of bees.

deviations from its linear progression due to the movement of the visual field are easily observable. A mirror placed above the bee's creeping compartment gives the observer a top view of the bee and of the visual field.

We used thirteen sizes of stripes. The larger ones we made by putting opaque

black paper between two pieces of glass. The finer ones were engraved on glass by Max Levy and Company of Philadelphia, and have the opaque areas filled with black printer's ink. The visual acuities furnished by these plates depend on their distance from the eye of the bee. On several occasions we measured this distance. It varies slightly from plate to plate and from bee to bee. The average is 17.3 mm. representing the distance from the center of the creeping bee's eye to the plate which has the pattern on it. In Table I is given the dimensions of the lines and spaces, the resulting visual angle, and the reciprocal of the visual angle representing the visual acuity. As in the case of ophthalmological practice a visual angle of 1 minute corresponds to a visual acuity of unity.

TABLE I.

Designations and Properties of Pattern Plates Used in the Experiments.

Distance of pattern from center of bee's eye is 17.3 mm.

Designation	Width of bar	Visual angle subtended by bar	Visual acuity $\times 10^4$
	mm.	min.	
A	19.0	2862.0	3.50
B	12.4	2136.0	4.68
C	9.4	1710.0	5.85
D	6.3	1200.0	8.33
E	4.7	912.0	10.96
F	3.2	630.0	15.87
G	1.27	252.0	39.68
H	0.847	168.0	59.52
I	0.635	126.0	79.37
J	0.508	100.8	99.21
K	0.423	84.0	119.0
L	0.363	72.0	138.9
M	0.318	63.0	158.7

The illumination of the striped pattern is varied in two ways. The 1000 watt lamp in dark room *B* may be placed in one of three fixed positions. Position I is 15 cm. from the opal plate in the wall between the two dark rooms; Position II is 50 cm.; Position III is 170 cm. from the opal plate. We made six separate calibrations of ten measurements each of the relative intensities emitted by the opal plate when illuminated by the lamp in each of the three positions. The relative intensities, putting Position I equal to 1 are:—Position II, 0.0862; Position III, 0.0140. The diaphragm controlling the area of the radiating opal plate gives a span of over 1:10. The smallest opening used is 2 cm. square; the largest, 8 cm. square. The diaphragm when used with the lamp in the three positions enables one to provide accurately and continuously an illumination covering a range of over 1:1000.

The size of the opening in the diaphragm can be read to 0.1 mm. by an engraved metal scale attached to one leaf in the diaphragm and a pointer wire attached to the other. We did not rely on the fact that the illumination on the striped pattern is proportional to the size of the radiating area in the diaphragm, but measured the actual brightness of the striped pattern when illuminated by eleven different areas of the diaphragm. The measured points, plotted as scale reading on the diaphragm against the logarithm of the resulting brightness, fall on a smooth curve. This we drew on a sufficiently large scale to use as a calibration curve.

2. The procedure in making the experiments is as follows. Active worker bees from our own colony are taken from in front of the hive. They are lightly anesthetised and the wings clipped. After recovery they are kept in wire cages, several to a cage, where they have access to plenty of honey. A number are then tested individually in the apparatus and from them are selected vigorous bees whose responses to the moving visual field are sharp. These are marked by differently colored spots of paint placed on the thorax or abdomen. They are kept warm, dry, and in the dark.

To make a measurement, a visual acuity plate is put into the movable frame, the light is turned on for a given illumination, and a bee is placed in the creeping compartment. The animal at once begins to creep and continues actively all the time it is in the compartment. First it creeps upward on the floor of the cell. Then it tries to crawl up the sides. This usually results in its dropping to the bottom, where it again crawls upward. If it can successfully creep up the side it may continue upside down on the top; in which case it arrives at the bottom and crawls up again on the floor of the compartment. Very often it drops from the roof, and starts its journey on the floor again. The fact that the floor is the only illuminated face of the compartment also helps to keep the bee creeping there. The result is that at very frequent intervals the animal creeps across the visual field. After it has moved about 2 cm. and its direction of progression is clear, the pattern plate is moved with a sharp but not too rapid motion. Depending on whether the bee responds or not, the intensity of the field is lowered or raised and the bee tested again. This is continued until the illumination is found at which the bee just responds to the movement.

It is an extraordinary sight to watch the precision with which a bee changes its direction of creeping under these conditions. If the pattern is moved, say, to the left, the creeping bee swings sharply to the right through an angle which is easily 45° and may be much more, and continues creeping in the new direction. During a single crawl of perhaps 10 cm. we have frequently made a bee alter its direction, right and left by moving the plate left and right, as many as four or five times in rapid succession.

In this connection we have made on observation often enough to be quite certain of its reality. When the plate is moved slowly, and the animal has established its new direction, if the plate is suddenly stopped the bee swings into a new direc-

tion as if the plate had suddenly been moved in the opposite direction. The appearance is reminiscent of the common nystagmus of vertebrates.

After the reading has been checked the bee is removed, placed in the cage in the dark, and another bee is tested in the same way. This is usually done with about ten bees. After all of them have been run through with one size of stripes, another plate is placed in the movable frame and the same bees tested on a different size of stripes. This procedure is continued at regular intervals during the day, in the course of which six or seven different sets of stripes are tested. By choosing the proper plates one can in this way cover very nearly the whole range of visual acuity with each bee. The next day, with a fresh batch of bees one can then investigate the whole range again, but with visual acuities in between those already tested. We have found this better than to test each bee to all the plates in one day, because the animals seem to respond more vigorously and survive better if they are tested at intervals of about an hour.

III.

Experimental Results.

1. The preliminary work for the experiments occupied over six months of almost daily effort before any of the measurements to be reported here were made. Beginning with the fact that bees respond to a movement in their immediate environment, we tried to elicit this response under controlled conditions which would make measurements possible. For nearly three months all kinds of devices for producing a controllable movement to which the animal would give a fixed response were tried without success, until the tilted compartment with the moving stripes below it solved the problem. The angle of tilt is quite important and is a compromise between a steep angle which causes a pronounced geotropic creeping and a gentle slope which keeps the bees from rolling down the incline. The gelatine covered creeping plate helps to increase the angle of tilt. The rest of the preliminary experiments were concerned with designing and testing different types of apparatus and different means of producing the visual pattern, with the results already indicated.

After all the conditions had been properly set, we began the final measurements early in June 1928. We made measurements with 91 normal animals in 9 days. The data are given in Table II where every measurement with every animal is recorded. The logarithms of the intensities are given instead of the intensities themselves for reasons which will become evident in a moment.

TABLE II.

Relation Between Visual Acuity and Illumination.

Visual acuity values from Table I; illumination in millilamberts.

Date	Bee No.	Logarithm of illumination for visual acuity												
		A	B	C	D	E*	F	G	H	I	J	K*	L	M
June 10, 1928	1					$\bar{1}$.280 $\bar{1}$.435						0.192 0.065		
	2					$\bar{1}$.310 $\bar{1}$.240								
	3					$\bar{1}$.439 $\bar{1}$.435						0.286 0.490		
	4					$\bar{1}$.265 $\bar{1}$.170								
	5					$\bar{1}$.378 $\bar{1}$.305						0.242 0.385		
	6					$\bar{1}$.319 $\bar{1}$.305								
	7					$\bar{1}$.233 $\bar{1}$.297						0.113 0.143		
	8					$\bar{1}$.310 $\bar{1}$.240								
	9					$\bar{1}$.330 $\bar{1}$.240						0.208 0.143		
	10					$\bar{1}$.403 $\bar{1}$.147								
June 11, 1928	11				$\bar{2}$.904			$\bar{1}$.771		$\bar{1}$.970			0.610	
	12				$\bar{1}$.040			$\bar{1}$.868		0.050			0.550	
	13				$\bar{2}$.941			$\bar{1}$.817		0.020			0.463	
	14							$\bar{1}$.689		$\bar{1}$.955			0.524	
	15				$\bar{1}$.096			$\bar{1}$.849		0.072			0.639	
	16							$\bar{1}$.809		0.050				
	17				$\bar{2}$.623			$\bar{1}$.745		$\bar{1}$.988			0.385	
June 12, 1928	18	$\bar{2}$.420	$\bar{2}$.465	$\bar{2}$.778	$\bar{1}$.074	$\bar{1}$.280 $\bar{1}$.314	$\bar{1}$.384							
	19	$\bar{2}$.324	$\bar{2}$.769	$\bar{2}$.825	$\bar{1}$.148	$\bar{1}$.265 $\bar{1}$.415								
	20		$\bar{2}$.299		$\bar{1}$.023	$\bar{1}$.118 $\bar{1}$.127								
	21	$\bar{3}$.915	$\bar{2}$.373	$\bar{2}$.730	$\bar{2}$.994	$\bar{1}$.319 $\bar{1}$.415	$\bar{1}$.325							
	22	$\bar{2}$.262	$\bar{2}$.681	$\bar{2}$.948	$\bar{1}$.057	$\bar{1}$.259 $\bar{1}$.343								
	23	$\bar{2}$.469	$\bar{2}$.382	$\bar{2}$.787	$\bar{1}$.136	$\bar{1}$.236 $\bar{1}$.218	$\bar{1}$.495							
	24	$\bar{2}$.444	$\bar{2}$.821	$\bar{2}$.809	$\bar{1}$.013	$\bar{1}$.307 $\bar{1}$.175	$\bar{1}$.368							

	25	$\bar{2}$.506	$\bar{2}$.692		$\bar{1}$.020	$\bar{1}$.389	$\bar{1}$.476	$\bar{1}$.386								
	26	$\bar{2}$.190	$\bar{2}$.713	$\bar{2}$.865	$\bar{1}$.091	$\bar{1}$.393	$\bar{1}$.155	$\bar{1}$.526								
	27	$\bar{2}$.293	$\bar{2}$.858	$\bar{2}$.805	$\bar{1}$.181	$\bar{1}$.393	$\bar{1}$.047	$\bar{1}$.047								
June 13, 1928	28				$\bar{1}$.185							0.175			0.639	1.352
	29				$\bar{1}$.044							0.095			0.475	0.930
	30				$\bar{1}$.035							0.114			0.550	1.300
	31				$\bar{2}$.913							0.098			0.332	1.040
	32				$\bar{2}$.910							0.127			0.406	1.180
	33				$\bar{1}$.015							0.010			0.652	0.992
	34				$\bar{1}$.010							0.150			0.530	1.349
	35				$\bar{1}$.140							0.053			0.600	1.349
	36				$\bar{2}$.926							0.086			0.580	1.240
	37				$\bar{2}$.865											
June 14, 1928	38							$\bar{1}$.462	$\bar{1}$.695	$\bar{1}$.740	$\bar{1}$.895		0.227	0.320		
	39							$\bar{1}$.341		$\bar{1}$.870			0.265	0.350		
	40							$\bar{1}$.314	$\bar{1}$.632	$\bar{1}$.809	$\bar{1}$.997		0.190	0.181		
	41							$\bar{1}$.495		0.028			0.254			
	42							$\bar{1}$.677	$\bar{1}$.836	$\bar{1}$.980	0.041		0.265			
	43							$\bar{1}$.578	$\bar{1}$.785	$\bar{1}$.910	0.020		0.198	0.308		
	44							$\bar{1}$.562	$\bar{1}$.774	$\bar{1}$.952	$\bar{1}$.980		0.257	0.278		
	45							$\bar{1}$.368		$\bar{1}$.850						
	46							$\bar{1}$.533	$\bar{1}$.880	$\bar{1}$.799	$\bar{1}$.982		0.294	0.332		
	47							$\bar{1}$.283	$\bar{1}$.774	$\bar{1}$.886	$\bar{1}$.852		0.249	1.908		
	48							$\bar{1}$.593		$\bar{1}$.952	0.080		0.246	0.580		
June 15, 1928	49														0.610	1.072
	50									$\bar{1}$.909		0.208			0.595	1.102
	51		$\bar{2}$.713							$\bar{1}$.817		0.086			0.367	1.124
	52		$\bar{2}$.793							$\bar{1}$.870		0.175			0.586	1.301
	53		$\bar{2}$.988							0.051		0.139			0.482	1.185
	54		$\bar{2}$.606							$\bar{1}$.809		0.024			0.542	1.230

* In Column E the values to the left were made with the lamp in Position III; the values to the right with the lamp in Position II. In Column K, the left values are with the lamp in Position II, the right values in Position I.

TABLE II.—*Continued.*

Date	Bee No.	Logarithm of illumination for visual acuity												
		A	B	C	D	E*	F	G	H	I	J	K*	L	M
June 15, 1928	55		$\bar{2}$.762						$\bar{1}$.945		0.146		0.534	1.140
	56		$\bar{2}$.527						$\bar{1}$.817		0.086		0.378	
	57										0.107		0.738	1.388
	58		$\bar{2}$.623						$\bar{1}$.955		0.071		0.660	1.218
June 16, 1928	59	$\bar{2}$.373		$\bar{1}$.060			$\bar{1}$.452	$\bar{1}$.768		0.058				1.080
	60	$\bar{2}$.008		$\bar{2}$.713			$\bar{1}$.610	$\bar{1}$.710		0.022				1.145
	61	$\bar{2}$.350		$\bar{2}$.713			$\bar{1}$.296	$\bar{1}$.858		0.080				1.230
	62	$\bar{2}$.206		$\bar{2}$.963			$\bar{1}$.402							1.310
	63	$\bar{2}$.420		$\bar{2}$.940			$\bar{1}$.386	$\bar{1}$.777		0.008				1.245
	64	$\bar{2}$.465		$\bar{2}$.976			$\bar{1}$.300	$\bar{1}$.946		0.090				1.124
	65	$\bar{2}$.382		$\bar{2}$.879			$\bar{1}$.545	$\bar{1}$.435		$\bar{1}$.885				0.930
	66	$\bar{2}$.455		$\bar{2}$.870			$\bar{1}$.509	$\bar{1}$.798		$\bar{1}$.963				0.902
	67	$\bar{2}$.587		$\bar{2}$.623			$\bar{1}$.170	$\bar{1}$.722		0.016				1.185
	68	$\bar{2}$.587		$\bar{2}$.920			$\bar{1}$.495	$\bar{1}$.790		$\bar{1}$.828				1.388
	69	$\bar{2}$.883		$\bar{2}$.868			$\bar{1}$.380	$\bar{1}$.817		$\bar{1}$.970				1.100
	70	$\bar{2}$.018		$\bar{2}$.730			$\bar{1}$.368	$\bar{1}$.828		$\bar{1}$.980				1.167
June 18, 1928	71	$\bar{2}$.161	$\bar{2}$.505		$\bar{1}$.150				$\bar{1}$.930		0.092		0.534	
	72	$\bar{3}$.974	$\bar{2}$.623		$\bar{2}$.987				$\bar{1}$.768		$\bar{1}$.980		0.406	
	73	$\bar{2}$.135	$\bar{2}$.324		$\bar{1}$.132				$\bar{1}$.885		0.100		0.345	
	74	$\bar{2}$.465	$\bar{2}$.299		$\bar{1}$.301				$\bar{1}$.990		0.020		0.482	
	75	$\bar{2}$.527	$\bar{2}$.685		$\bar{2}$.904				$\bar{1}$.965		0.080		0.425	
	76	$\bar{2}$.373	$\bar{2}$.825		$\bar{1}$.150				$\bar{1}$.970		0.208		0.716	
	77	$\bar{2}$.190	$\bar{2}$.713		$\bar{1}$.060				$\bar{1}$.930		0.100		0.258	
	78	$\bar{3}$.885	$\bar{2}$.838		$\bar{1}$.234				$\bar{1}$.828		0.035		0.534	

	79	$\bar{2}$.420	$\bar{2}$.623		$\bar{1}$.210				$\bar{1}$.880		0.071		0.444	
	80	$\bar{2}$.161	$\bar{2}$.495		$\bar{1}$.069				$\bar{1}$.850				0.534	
	81	$\bar{3}$.820	$\bar{2}$.642		$\bar{2}$.890				$\bar{1}$.734		$\bar{1}$.904		0.550	
	82		$\bar{2}$.746		$\bar{1}$.176				$\bar{1}$.888		0.139		0.639	
	83	$\bar{2}$.219	$\bar{2}$.778		$\bar{1}$.301				$\bar{1}$.912		0.100		0.500	
June 19, 1928	84			$\bar{1}$.060		$\bar{1}$.578		$\bar{1}$.886		$\bar{1}$.980		0.192		1.222
	85			$\bar{2}$.930		$\bar{1}$.358		$\bar{1}$.790		0.004		0.345		1.326
	86			$\bar{2}$.865		$\bar{1}$.195		$\bar{1}$.750		$\bar{1}$.946		0.444		1.199
	87			$\bar{2}$.941		$\bar{1}$.365		$\bar{1}$.798		$\bar{1}$.970		0.118		1.109
	88			$\bar{1}$.019		1.262		$\bar{1}$.695		$\bar{1}$.955		0.236		1.180
	89			$\bar{1}$.132				$\bar{1}$.849		0.080		0.406		1.026
	90			$\bar{2}$.998		$\bar{1}$.419		$\bar{1}$.817		$\bar{1}$.895		0.303		1.124
	91			$\bar{2}$.917		$\bar{1}$.098		$\bar{1}$.828		0.100		0.236		1.230
Average.....		$\bar{2}$.300	$\bar{2}$.639	$\bar{2}$.881	$\bar{1}$.051	$\bar{1}$.294	$\bar{1}$.422	$\bar{1}$.781	$\bar{1}$.889	$\bar{1}$.994	0.093	0.257	0.521	1.177

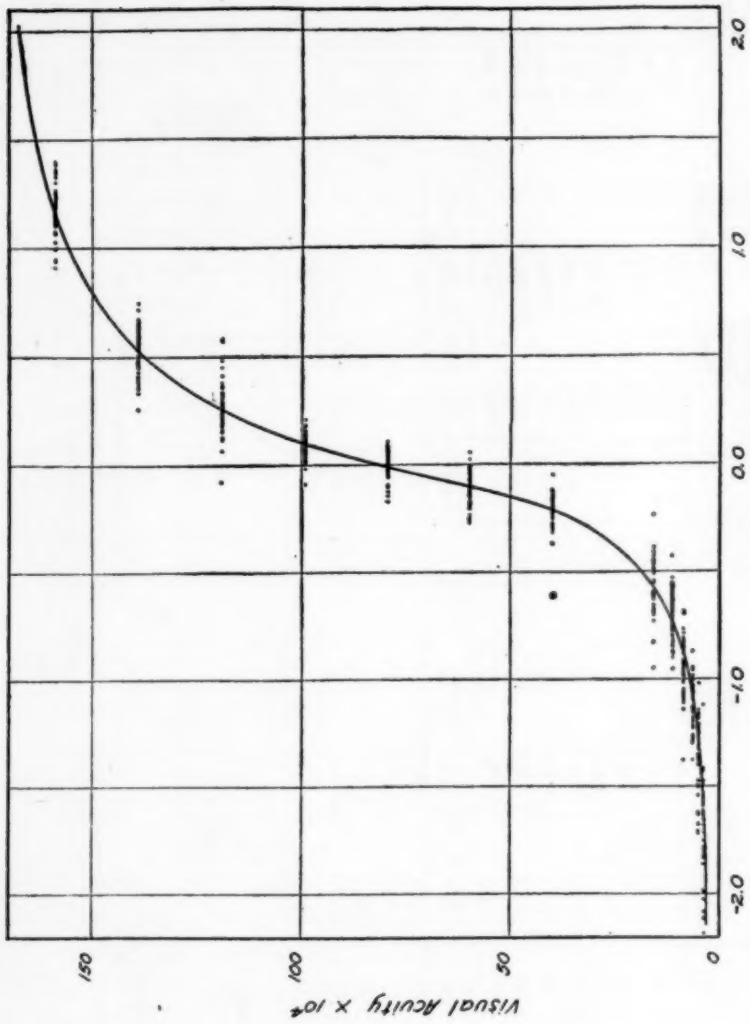

FIG. 2. Relation between visual acuity and illumination. An effort has been made to represent with a dot each measurement with each bee. This is manifestly impossible in the places where the measurements come very close together. The plot therefore shows the general way in which the measurements run. The full details are given in Table II. The curve is drawn through the mean values of the measurements.

2. The way in which in Table II the different individuals vary in the minimum intensity for a given visual acuity is significant. The range of variation is greatest at low visual acuities; it decreases as the visual acuity increases; and at the highest visual acuities, it again becomes larger. In Fig. 2 the same data are presented graphically. At once the reason for the curious distribution of the variation becomes clear.

The relation between visual acuity and the logarithm of the illumination is sigmoid, much as for the human eye. It follows that at low visual acuities a very small difference in visual acuity corresponds to a large difference in intensity; at the middle range, visual acuity is almost directly proportional to log I, the curve being quite steep; finally at high visual acuities a condition prevails similar to that at low acuities. At the two ends a very slight individual difference in the animal shows itself in a large difference in minimum illumination, whereas the reverse is true in the middle ranges. This is shown by the closeness with which the individual points at the lower end adhere to the curve drawn through the average values of the data.

3. The shape of the curve in Fig. 2 indicates that the experiments cover the whole range of visual acuity of which the bee is capable. The maximum value lies between 0.016 and 0.017. This is below the visual acuity of the human eye at the lowest perceptible illuminations. Our maximum visual acuity is about 1.5; our minimum is about 0.03 (Koenig, 1897; Hecht, 1927–28). Incredible as it may seem, the bee's greatest capacity for the optical resolution of its environment is never better than ours is at our worst.

The disparity in the visual acuity of our eyes and those of the bee is even greater than this and is brought out by considering the visual acuities at the same illuminations. The maximum visual acuity in both cases occurs at very nearly the same intensities of illumination and corresponds to a brightness of between 50 and 100 millilamberts. Our maximum here is about 1.5; the bee's maximum is 0.017. In other words we can resolve the environment about 100 times better than a bee can. That this low value is no laboratory product is borne out by the experiments of Baumgärtner (1928) in which bees on the wing in the field were shown to have a similar, almost negligible, form discrimination. Baumgärtner, using v. Frisch's conditioned-reflex

method, found that in broad daylight a bee can just recognize a 2 cm. square at a distance of about 40 cm. This corresponds to a visual angle of a little less than 3°, and a visual acuity of about 0.006. This is even lower than our values, but still of the same order of magnitude.

4. The intensity range over which the bee and the human eye function in relation to visual acuity is such that for each the maximum visual acuity is about 50 times the minimum. In both cases the maximum occurs at about the same intensity of illumination, 100 ml. The lowest visual acuity for the bee is 0.00035 and occurs at 0.020 ml. At intensities below this the animals wander all over the creeping compartment and do not respond to the movements of even larger patterns than here recorded. This is an intensity range of about 3.5 logarithmic units. The same span of visual acuity is spread by our eye over a range of over 7 logarithmic units of which 2.5 are controlled by the rods and 4.5 by the cones. Our eyes thus function over intensities 3.5 log units lower than those of the bee.

IV.

Anatomical Basis of Visual Acuity.

1. In spite of these differences in magnitude and range between the visual acuity of the human eye and that of the bee's eye, the relation between visual acuity and illumination is essentially the same in the two. The resolving power of the bee's eye is low at low illuminations and increases with the logarithm of the illumination up to a maximum, in the same S-shaped way as the human eye. The same kind of theoretical treatment is therefore indicated.

Differences in resolving power such as are found here mean differences in the distances which separate the centers of the receiving elements. The data, then, if interpreted naïvely would require that the ommatidia vary in their distance apart, assuming different positions depending on the intensity. This obviously cannot be true because the eye of the bee is a fixed organ and does not change either its shape or its size under illumination. The data must therefore be interpreted in some other way, the requirement being to secure a functional separation of ommatidia which are structurally fixed.

To solve this problem it is supposed that the receptor elements in the ocular mosaic do not all possess the same threshold, but that the threshold varies among the ommatidia as does any other characteristic in a population. This then works out so that at low illuminations only a few ommatidia are functional. Since these are distributed at random, they will be far apart and will give the same result functionally as if there were no receiving structures between them. As the illumination increases, more and more ommatidia become functional; the distance between functional elements becomes smaller; and the resolving power becomes greater. This continues until an illumination is reached which is above the threshold of the most insensitive ommatidium. All the elements will then be functional, and no further increase in visual acuity can take place.

It is apparent that such an explanation is in general agreement with the data, and could be formulated in strictly quantitative terms for comparison with the data. However, before this can be done in a significant manner it is necessary to examine more closely the nature of the data in their relation to the structure of the eye.

2. In the experiments the animals were measured as they crawled in directions nearly parallel to the length of the stripes. The visual pattern is therefore registered across the long axis of the eye. Let us neglect for a moment the fact that since the plane of the pattern is roughly perpendicular to the long axis of the eye, the successive bars in the pattern will occupy a decreasingly smaller visual angle. At an illumination when all the elements are functional, the maximum visual acuity will then occur when a horizontal row of elements receives light, and an adjacent row receives no light, and so on. The size of the smallest perceptible pattern will correspond to the visual angle which separates the centers of two adjacent elements.

If the elements were all the same angular size and were evenly distributed on the surface of the eye, the maximum visual acuity would be perceptible all over the eye. But the elements in the bee's eye are not uniform in angular dimension. Then the maximum visual acuity can obviously be perceptible only at that point on the eye where the population density of visual elements is at a maximum, and the resulting angular separation a minimum. In the very nature of our measurements the visual acuity corresponding to any illumination is

always the maximum visual acuity at that intensity. Therefore at any illumination, no matter how many functional elements it represents, the visual acuity, as given, is determined at that area on the eye where the population density of functional elements is greatest. This is a fundamental concept in understanding the data. Fortunately it may be tested experimentally.

TABLE III.

Angle Subtended by Walls of Ommatidia in Longitudinal Section.

Ommatidial density in different parts of eye. Data obtained graphically from Abb. 29 in Baumgärtner (1928).

Ommatidium No.	Angle	Ommatidia per unit angle
	degrees	
5	2.22	0.45
10	1.89	0.53
20	1.56	0.64
30	1.41	0.71
35	1.33	0.75
40	1.22	0.81
45	1.13	0.89
50	1.02	0.98
55	0.94	1.07
60	0.88	1.13
65	0.88	1.13
70	0.92	1.09
80	1.02	0.98
90	1.10	0.91
100	1.19	0.84
110	1.32	0.76
120	1.52	0.66
130	1.97	0.51
140	3.57	0.28

The measurements as plotted in Fig. 2 show that the maximum visual acuity of which the bee is capable at the highest illuminations is very nearly 0.017. This corresponds to a visual angle of 0.98°. Since at these illuminations all the ommatidia are functional, this experimentally determined, minimal, angular separation should correspond to the smallest vertical separation between adjacent ommatidia as determined anatomically. Baumgärtner (1928) has

recently made a histological study of the bee's eye, and has measured the angular separation of adjacent ommatidia in different sections of the eye. In vertical section the angular separation increases about four times from center to periphery. The smallest separation is near the middle, in the lower half of the eye and includes between 20 and 25 elements in vertical section. Its value lies between 0.9° and

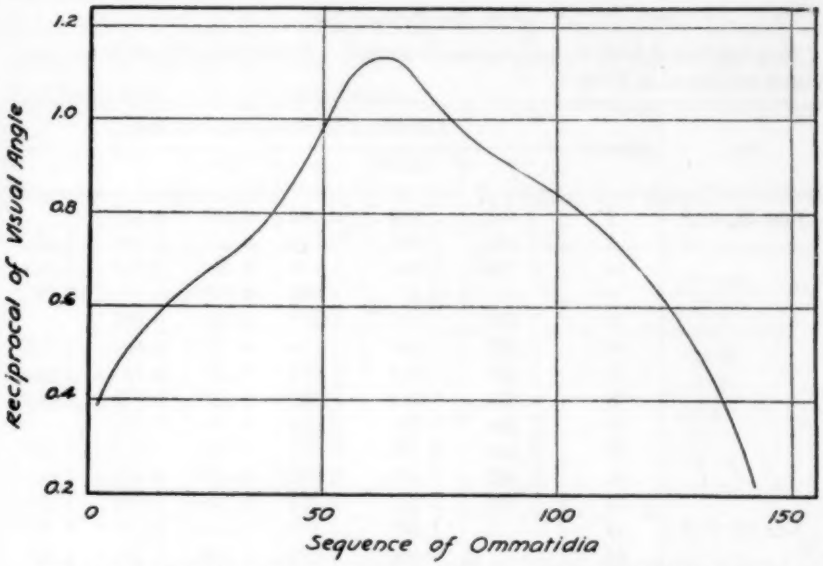

FIG. 3. Morphological distribution of visual acuity in a longitudinal section of the bee's eye. Data taken from Abb. 29 in Baumgärtner's (1928) paper. The abscissas are the ommatidia in sequence beginning with the ventral ommatidium and going dorsally. The ordinates are the reciprocals of the angle made by the walls of the corresponding ommatidia. The ordinates are eqivalent to the relative number of ommatidia in a unit angle in different parts of the eye.

1.0°. This agreement between experimental and morphological findings indicates that visual acuity is determined by the region of maximum population density.

3. The evidence for this conclusion becomes even better when the morphological findings are examined in detail. Baumgärtner measured the angular separation of the two walls of each ommatidium in the longitudinal section of the eye. Table III contains the data,

taken graphically from Fig. 29 of Baumgärtner's paper, which describe his findings with 8 such sections. The data are plotted in Fig. 3. Here the abscissas are the ommatidia numbered in serial order beginning at the bottom of the eye; the ordinates are the reciprocals of

TABLE IV.

Relation Between Visual Acuity and Illumination in Bees With the Central Area of the Eye Painted Out.

Non-functional area is one-quarter of normal. Illumination in millilamberts; visual acuities as in Table I.

Date	Animal No.	Logarithm of illumination for visual acuity					
		F	G	H	I	J	K
June 28, 1928	1	$\bar{1}$.493	$\bar{1}$.948	0.092	0.165	0.610	
	2	$\bar{1}$.666	$\bar{1}$.942	0.016	0.127	0.406	1.222
	3	$\bar{1}$.558	$\bar{1}$.919	$\bar{1}$.960	0.254	0.345	1.283
	4		$\bar{1}$.817	$\bar{1}$.988	0.195		
	5	$\bar{1}$.613	$\bar{1}$.673	$\bar{1}$.983	0.202	0.693	
	6	$\bar{1}$.502	$\bar{1}$.900	0.028	0.242	0.603	1.128
	7	$\bar{1}$.593	$\bar{1}$.984	0.058	0.213	0.534	1.065
	8	$\bar{1}$.423	$\bar{1}$.774	$\bar{1}$.959	0.120	0.438	1.088
	9	$\bar{1}$.240	$\bar{1}$.793	0.060	0.240	0.364	
	10	$\bar{1}$.704	0.006	0.105	0.192	0.479	1.339
	11	$\bar{1}$.602	$\bar{1}$.890	0.040	0.170	0.450	1.185
	12	$\bar{1}$.550	$\bar{1}$.877	0.024	0.146	0.201	1.300
June 29, 1928	13		$\bar{1}$.801	0.010	0.172	0.359	1.153
	14		$\bar{1}$.946	0.038	0.195	0.550	1.205
	15		$\bar{1}$.867	0.030	0.152	0.503	
	16		$\bar{1}$.990	0.111	0.215	0.610	1.121
	17		$\bar{1}$.921	0.072	0.159	0.163	1.236
	18		$\bar{1}$.928	0.090	0.195		
	19		$\bar{1}$.817	$\bar{1}$.959	0.133	0.367	1.321
	20		$\bar{1}$.885	0.004	0.150	0.463	1.191
	21		$\bar{1}$.863	$\bar{1}$.988	0.127	0.118	
Averages.....		$\bar{1}$.540	$\bar{1}$.883	0.029	0.179	0.435	1.203

the angular separation of adjacent ommatidia, and therefore represent the density of ommatidia distribution in vertical section. Attention is called particularly to the region of great ommatidia density in the lower half of the eye where occur the elements of smallest angular separation already referred to. The arrangement is not unlike the

concentration of the smallest retinal elements which form the fovea of mammals.

What should happen if this region of high ommatidial density were rendered functionless? Clearly, the maximum visual acuity should be decreased, since it would have to be mediated by the region of the eye with a lower concentration of visual elements. Moreover the whole relation between visual acuity and illumination should become depressed, in that the visual acuity at any illumination should be lower than normal and dependent on the extent of the fovea which has been rendered nonfunctional.

TABLE V.

Comparison Between Visual Acuity of Normal Bees and of Bees With Central Part of Eye Painted Out.

Logarithm of illumination	Visual acuity × 10^4		Painted / Normal
	Normal	Painted	
$\bar{1}$.540	20.6	15.9	0.77
$\bar{1}$.883	58.3	39.7	0.68
0.029	87.2	59.5	0.68
0.179	110.4	79.4	0.72
0.435	133.9	99.2	0.74
1.203	159.0	119.0	0.75

We made experiments in which a spot of black paint was placed in the center of each eye. It is, of course, difficult to place such a patch of paint with very great accuracy, and to do it uniformly from eye to eye. We tried to cover about a quarter of the area of the eye. In vertical section such a patch extends over about half the ommatidia and eliminates those elements falling between ommatidium No. 35 and No. 100. With the fovea removed down to about ommatidium No. 35, the maximum density of elements is between 0.75 and 0.80, which corresponds to an angular separation of 1.3°. Since the minimum angular separation for the normal bee is very nearly 0.9°, the maximum visual acuity of the bees with the fovea painted out should be between three-quarters and two-thirds of normal at the highest illuminations. Moreover, since, as is apparent from Fig. 3, the ommatidial density decreases in a roughly uniform manner from the center

to the periphery, the visual acuities at any other illuminations should be correspondingly lower.

We made two series of measurements in the usual way in which the relation was determined between visual acuity and illumination for

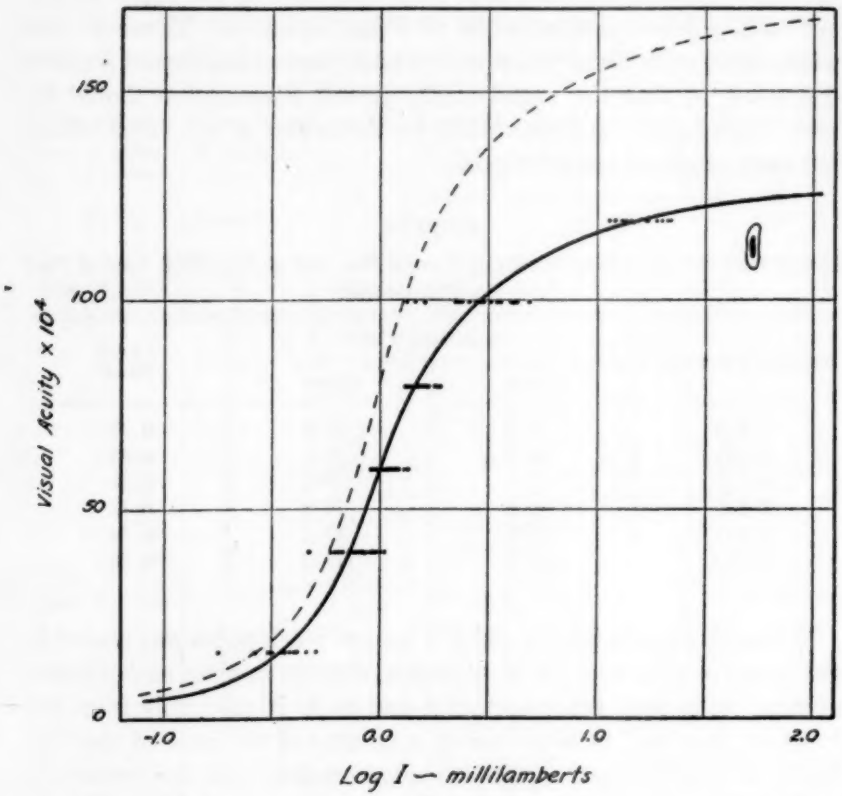

FIG. 4. Relation between visual acuity and illumination for bees with the central part of the eye painted out as shown in the figure. The points are individual measurements. The broken curve is the normal relation taken from Fig. 2. The full curve is made from the normal curve by multiplying its ordinates by 0.75.

bees with a central spot of paint on each eye. One series contained 12 animals; the other 9 animals. The results are given in Table IV in which the individual measurements are recorded as before. Table V

gives the average values of the intensities, the corresponding visual acuities for the painted bees, and for normal bees as taken from Fig. 2. The last column gives the ratios of the visual acuity of the painted eye to that of the normal eye at the same intensity. The ratio is obviously constant.

The data are also presented in Fig. 4, in which the two series are not treated separately. The broken curve in Fig. 4 is the normal curve taken from Fig. 2. The solid curve through the points is this same normal curve with its ordinates multiplied by 0.75. This agreement with expectation is surprisingly good, and shows that visual acuity determination in the bee's eye is a regional function, and depends on the utilization of the part of the eye containing the densest population of active elements.

It is significant that the region of densest ommatidia population is in the lower half of the eye, because the lower half of the eye is concerned with our measurements. This is apparent from the relative positions of the eye and the pattern. However, we tested it experimentally as well by painting out the upper half of the eye. Such a procedure seems to have no effect on the general behavior of the bee nor on its visual acuity.

4. Our measurements of visual acuity were so conducted that the pattern was registered on the long dimension of the eye. Consider an illumination when only a fraction, say one-ninth, of the visual elements are functional. Since the thresholds are distributed at random, any two functional elements are separated by two non-functional ones. Assuming for the moment that the mosaic is uniform, the angular distance between the functional elements will now be three times as great as in the fully functional eye. If the visual pattern varied in many directions, the resulting visual acuity would then be a third of its maximum value. But the present pattern is a uni-directional one, and can be resolved only along the vertical axis of the eye. The visual acuity corresponding to the present situation will then be determined by the projection on the long axis of the visual angle between any two functional elements. Moreover the ocular mosaic is not uniform. The visual acuity will then be determined by the apparent vertical separation of functional ommatidia in the region of greatest density.

This region of maximum density is always small, as Fig. 3 shows, and will contain only a few functional elements for which this vertical separation corresponds to the maximum visual acuity at that illumination. Undoubtedly a certain minimal number of such elements are required for the reception of the stimulus. Since the eye is very

TABLE VI.

Relation Between Visual Acuity and Illumination for Animals With One-half the Eye Painted Out.

Illumination in millilamberts; visual acuity as in Table I.

Date	Animal No.	Logarithm of illumination for visual acuity					
		E	F	G	H	I	J
June 23, 1928	1	$\bar{1}$.384	$\bar{1}$.384	$\bar{1}$.734	0.170	0.444	
	2	$\bar{1}$.578	$\bar{1}$.716	0.071	0.184	0.679	1.018
	3	$\bar{1}$.563	$\bar{1}$.710	0.032	0.225	0.518	1.034
	4	$\bar{1}$.614	$\bar{1}$.675	0.050	0.213	0.695	1.178
	5	$\bar{1}$.314	$\bar{1}$.434	$\bar{1}$.778	0.061	0.574	1.310
	6	$\bar{1}$.519	$\bar{1}$.660	0.076	0.206	0.580	0.863
	7	$\bar{1}$.477	$\bar{1}$.549	0.028	0.282	0.518	1.072
	8	$\bar{1}$.463	$\bar{1}$.675	$\bar{1}$.930	0.231	0.667	
	9	$\bar{1}$.661	$\bar{1}$.777	0.001	0.170	0.664	1.193
	10	$\bar{1}$.608	$\bar{1}$.675	$\bar{1}$.965	0.050	0.464	1.201
	11	$\bar{1}$.402	$\bar{1}$.648	$\bar{1}$.939			
June 25, 1928	12	$\bar{1}$.419	$\bar{1}$.608	0.050	0.208	0.325	1.018
	13	$\bar{1}$.470	$\bar{1}$.614	$\bar{1}$.817	$\bar{1}$.970	0.438	1.218
	14	$\bar{1}$.343	$\bar{1}$.777	$\bar{1}$.863	0.138	0.500	1.172
	15	$\bar{1}$.545	$\bar{1}$.552	$\bar{1}$.969	0.120	0.708	1.026
	16	$\bar{1}$.533	$\bar{1}$.638	$\bar{1}$.828	0.220	0.345	
	17	$\bar{1}$.402	$\bar{1}$.722	0.004	0.246	0.514	1.153
	18			$\bar{1}$.963		0.565	
	19	$\bar{1}$.384	$\bar{1}$.648	$\bar{1}$.912	0.110	0.530	
Averages.....		$\bar{1}$.482	$\bar{1}$.637	$\bar{1}$.948	0.165	0.540	1.112

nearly symmetrical in its two halves, there will be a symmetrical distribution of these few critical elements on either side of the central vertical axis. If now one side of the eye were rendered functionless, this should at once reduce the number of densely grouped elements by half. To get the same number of functional elements as before in order to resolve the pattern, elements which are more widely separated

would have to be drawn on, and at once the visual acuity should be decreased.

We made two series of experiments which test this; one with 11 bees and the other with 8 bees. In these we painted out approximately half of each eye, the dividing line being along the vertical axis. The results are given in Tables VI and VII, the latter containing the ratios of the visual acuities here obtained with those found for the normal bee at the same illumination. The ratios are seen to be nearly the same. This is brought out by Fig. 5 where the data are presented graphically. Through the experimental points there is drawn a con-

TABLE VII.

Comparison Between Visual Acuity of Normal Bees and of Bees With Anterior Half of Eyes Painted Out.

Logarithm of illumination	Visual acuity $\times 10^4$		Painted / Normal
	Normal	Painted	
$\bar{1}.482$	17.8	11.0	0.62
$\bar{1}.637$	26.4	15.9	0.60
$\bar{1}.948$	70.9	39.7	0.56
0.165	108.6	59.5	0.55
0.540	140.1	79.4	0.57
1.112	157.9	99.2	0.63

tinuous curve whose ordinates are 0.62 of the values for the normal, unpainted eye.

It is therefore apparent that the reduction of visual acuity as the result of painting out half the eye is of the kind which should occur if the resolution of the visual pattern is determined by the vertical separation of the functional ommatidia in the region of maximum density.

We made several series of experiments in which the functional part of the eye was further reduced. As is to be expected, the resulting visual acuity function becomes more and more depressed. The data are not given, because they are of no quantitative significance, since the actual part of the eye which remains unpainted is not definable in any quantitative terms as it decreases in size.

5. The conclusion to which we have just arrived, as well as the

facts which have led to it result in a minor paradox. In computing in Table I the visual angle corresponding to each of the pattern plates we considered the angle subtended by the two edges of a bar, one side

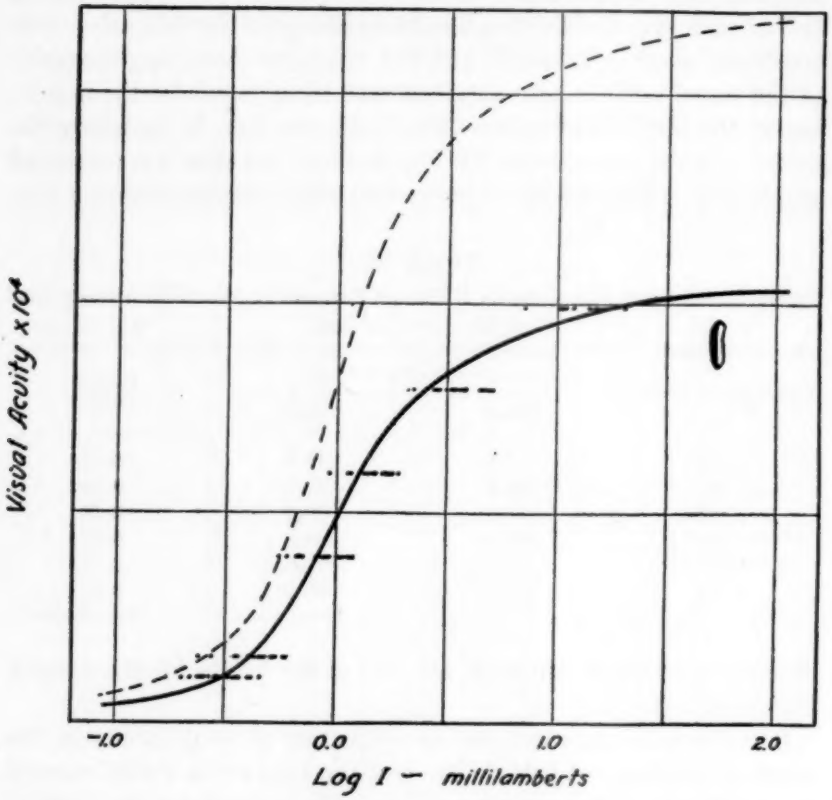

FIG. 5. Relation between visual acuity and illumination for bees with the anterior half of the eyes painted out. The points are individual measurements. The broken curve is the normal curve of Fig. 2. The full curve is constructed from the normal by multiplying its ordinates by 0.62.

of the angle being the perpendicular from the center of the eye to the plate. The tangent of the visual angle is the width of the bar divided by the perpendicular distance of the eye from the plate.

If the plane of the plate were parallel to the long axis of the eye this treatment would correctly give the visual angle subtended at any part

of the eye. But since the plate is practically perpendicular to the long axis of the eye the visual angle subtended by a bar varies with the part of the eye which is concerned with the resolution of the pattern. The paradox then is that we have been comparing a visual angle measured on the plate at the perpendicular from the eye with an angular separation of ommatidia situated one-third of the way up the eye.

The paradox largely disappears when it is remembered that the head of the bee as it creeps is not rigid. Even at rest the head is not kept perpendicular to the creeping plane; and when the animal creeps the position and angle of tilt of its head vary considerably. For a good part of the time during creeping the visual angles as given in Table I probably represent the real angles. This is borne out by the coincidence between the anatomical visual angle and the experimental visual angle at the highest illuminations. This can hardly be considered fortuitous, especially when it is reinforced by the agreement of the experimental findings with those calculated on the basis of painting out portions of the eye. We have therefore retained the visual angles for the plates as given in Table I on the supposition that they are an appropriate designation of the resolving power of the eye at the intensities to which they apply

V.

Visual Acuity and Number of Elements.

1. Having considered the anatomical and structural basis of visual acuity in the bee's eye, we may now apply this information to a closer analysis of the relation between visual acuity and illumination as it concerns the number of elements functional,—an analysis postponed from the previous section. Off hand it would seem that, since visual acuity is determined by the vertical distance between elements in the region of maximum ommatidial density, the curve in Fig. 2 represents the number of functional elements in the vertical axis of the fovea corresponding to any illumination. This would be true if the ocular mosaic were uniform. Since however, the angular separation between adjacent ommatidia is not constant, the precise way in which this

varies must be considered in the conversion of visual acuity into number of ommatidia functional.

In Fig. 6 we have plotted part of the lower half of the bee's eye, ommatidium for ommatidium, beginning with ommatidium No. 25 and ending with No. 70 which is just in the center of the eye. The linear distance is in terms of ommatidial angle as taken from Baumgärtner's average curve given in Table III and in Fig. 3. This region covers a total angle of about 50° and therefore includes the whole range of visual angle encountered in the measurements.

The ommatidia lying in the center of the foveal region, that is Nos. 56 to 70, have practically the same visual angle, No. 56 has an angle of 0.92°; those in between gradually decrease to No. 63 whose angle is

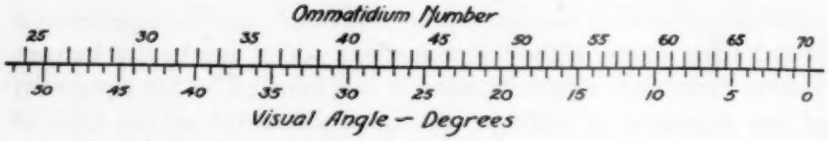

FIG. 6. Graphic representation of the method of finding the number of ommatidia included in a given visual angle. The lower scale is a linear scale of visual angles. The upper scale gives the angle subtended by the individual ommatidia, beginning with No. 70 at zero angle.

0.88° and then increase again to No. 70 which also has an angle of 0.92°. This variation of about 5 per cent may be neglected in view of the almost 100 per cent increase in visual angle shown by ommatidium No. 25. The significance of this middle region is that since it is the area of maximum population density the chance is greatest of its being the location of the most sensitive ommatidium. Therefore even at the lowest illumination, one functional element is most likely to be found here, and will continue to be here at all intensities. It may therefore be taken as the reference point from which to measure the vertical angular separation on the eye corresponding to any visual angle.

Which of the dozen or so elements in this practically uniform region of maximum density is the most sensitive one it is not possible to say. We have selected ommatidium No. 70, because the distance from it to any point on the lower half of the eye will always include the greatest

number of elements. It may be added that for the present purposes it matters only slightly whether we choose ommatidium No. 63 which is in the exact center of the fovea, or No. 70. The numerical results which we are after differ only insignificantly in the two circumstances, and the principle is the same.

Below the diagram of the ommatidia in Fig. 6, we have drawn a simple linear scale of visual angle, beginning with zero at the upper edge of ommatidium No. 70. Using these two scales, one can read off how many ommatidia in the vertical projection on the upper scale

TABLE VIII.

Relation Between Visual Angle, the Number of Ommatidia Included in the Angle, and the Number of Ommatidia Functional per Unit Angle.

Visual angle	Number of ommatidia included	Number of ommatidia functional in unit angle
degrees		
47.7	43.55	1.00
35.6	34.83	1.25
28.5	29.24	1.49
20.0	21.67	2.01
15.2	16.84	2.59
10.5	11.78	3.70
4.20	4.65	9.37
2.80	3.08	14.14
2.10	2.30	18.93
1.68	1.84	23.67
1.40	1.53	28.46
1.20	1.31	33.24
1.05	1.14	38.20

are included in a given visual angle on the lower scale. At the greatest visual angle of 47.7° there are included 43.55 ommatidia. This means that the angle formed at the center of the eye by the lines from the two functional ommatidia nearest together includes 43.55 ommatidia in its projection on the longitudinal meridian of the eye. It therefore follows that only 1 out of 43.55 ommatidia is functional. This of course is only in projection on the vertical meridian, and does not mean the number of ommatidia which actually separate the two functional ommatidia in a direct line connecting them on the surface of the eye. This real value is naturally a whole number, and not a

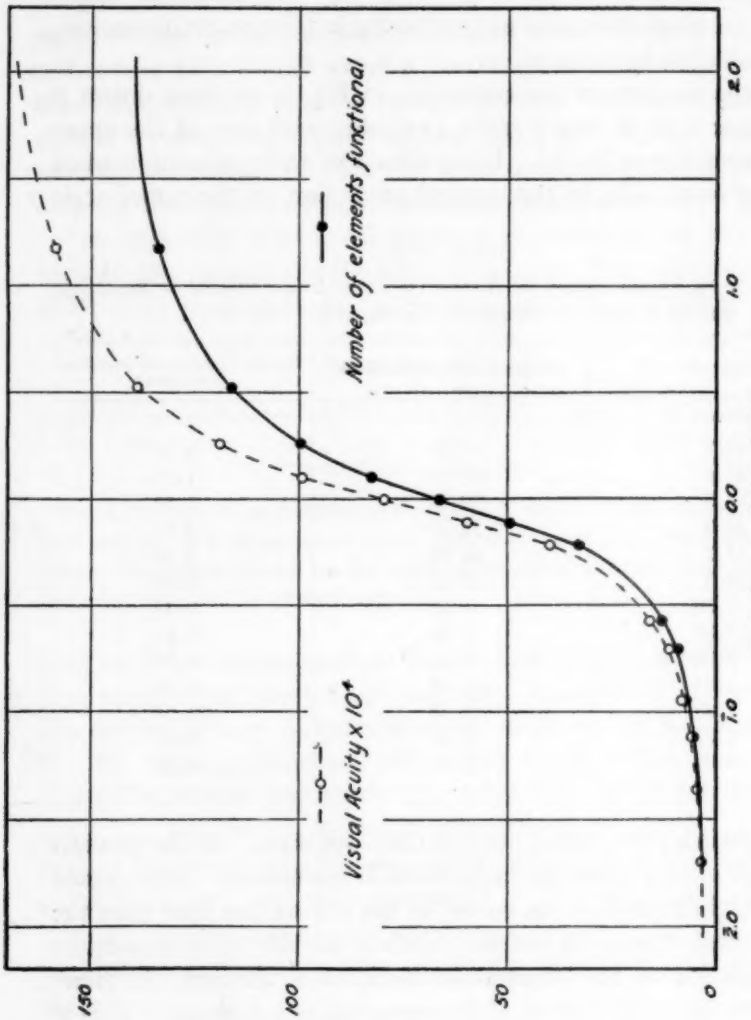

FIG. 7. Comparison between visual acuity and number of ommatidia functional in a given angular distance in their relation to the logarithm of the illumination. The relation between the number functional and log I is in the nature of an integral probability or distribution curve.

fractional one. The same procedure may be continued in order to determine the projected separation of the two functional ommatidia corresponding to any other visual angle.

The use of Fig. 6 in determining ommatidial density along the eye is a graphic method. Actually we determined these values arithmetically using exactly the same principle as described graphically. The values so secured are given in the second column of Table VIII. At a visual angle of 47.7°, there is functional 1 out of every 43.55 ommatidia. At a visual angle of 35.6° this will be 1 out of every 34.83

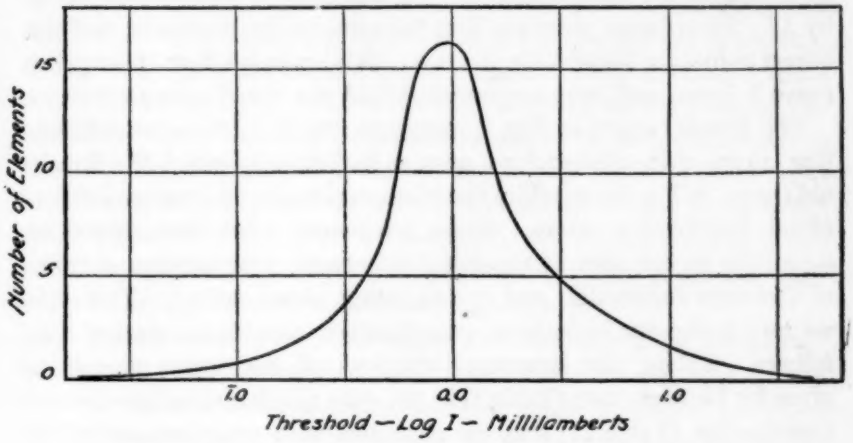

FIG. 8. Distribution of thresholds of the ommatidia in the bee's eye. The curve is the first differential of the number curve in Fig. 7, and is a differential probability or distribution curve.

ommatidia; or 1.25 out of every 43.55 ommatidia, if one considers the largest visual angle as the unit inside of which all the other angles must fall. In the same way all the other angles are computed with the results given in Column 3 of Table VIII.

We have thus made, in terms of the structure of the eye, a transformation from visual acuity data to the corresponding number of elements functional in a given distance on the vertical meridian of the eye. It is true that we do not have the real numbers but only the numbers in vertical projection. This is not without significance, as will be apparent presently. However, since the surface of the eye

is a hexagonal mosaic the real numbers are a simple geometric function of the numbers projected on the vertical meridian. The ratio of real distance to projected distance in such a mosaic is as $\sqrt{3}$ is to 1; so that any conclusions drawn for one holds for the other.

2. In order to show how these values of the number of functional elements are related to the visual acuity data from which they are derived, we have plotted both in Fig. 7. The upper curve is the visual acuity relation, the points being the averages from Table II. The lower curve is the number curve. So that the two might more readily be compared we have multiplied the ordinates of the number curve by 3.5; the ordinate units are thus the same in the two cases, and the lowest points are identical in the two. It is apparent that the number curve is lower, and more symmetrical than the visual acuity curve.

The number curve in Fig. 7 resembles the usual integral distribution curves of the statisticians, even as its first differential, the threshold curve, in Fig. 8 resembles the more commonly encountered differential distribution curves. These are indeed what they should be according to our idea of the relation between illumination, number of elements functional, and the resulting visual acuity. Therefore we may make our hypothesis quantitatively specific by stating it as follows. Taking the structural relations of the ocular mosaic as given by Baumgärtner (Table III), the data relating visual acuity and illumination (Table II) may be described with complete fidelity by assuming a distribution of the thresholds of the various ommatidia corresponding to the population curve of Fig. 8.

3. This distribution curve may be interpreted in two ways. So far we have tacitly assumed for the human eye and for the bee's eye that a given threshold is a permanent characteristic of a given element. The curve in Fig. 8 then represents the distribution of this characteristic in the population of ocular elements. For the human eye we even suggested a possible photochemical basis for this distribution (Hecht, 1927–28). However, one may conceive this situation purely in terms of probability. Assume that the threshold of a given element is not fixed, but can vary over the whole range included in Fig. 8. Then at any moment the probable distribution of elements over the entire threshold range is given by the curve in Fig. 8. This may therefore be used as the basis for visual acuity in precisely the same way as before.

We frankly incline toward the fixed threshold interpretation largely because it seems unlikely to us that a given cell can vary in its threshold from moment to moment over such a very large range of illuminations. However the matter may be tested experimentally by studying the dark adaptation of very small retinal areas in the human eye. If the increase in sensibility during dark adaptation for minute areas is as smooth and regular as for large areas then the threshold of a given element is most likely a permanent property.

It is apparent that the computations, experiments, and reasoning in Section IV and in a good part of the present section could have been avoided if we had merely assumed that the ocular mosaic is uniform, or had been ignorant of its being otherwise. Then the direct visual acuity curve in Fig. 2 could have been assumed as the integral distribution curve. Its first differential would have been considered as the frequency distribution of the thresholds which have to be assumed to account for the data. It is only because we wished to correlate structure and function that we performed the experiments in which various portions of the eye were painted out. The more extended analysis here presented then became necessary.

4. There remain some minor aspects of the data to be considered. Studies on other arthropods have shown that the pigment in and between the ommatidia migrates, and that this migration is controlled by the illumination. The position of the pigment between the ommatidia may affect the degree to which these are isolated from one another, and may thus influence visual acuity. It is difficult to estimate the magnitude of this factor because we know of no data for the bee's eye. Bennitt's (1924) work, however, would indicate that it may be comparatively negligible because, in the arthropods which he studied, the migration begins at quite low illuminations, and is complete over a very restricted increase in intensity. If the bee's eye behaves in a similar manner, pigment migration would hardly enter to distort the relationship shown in Fig. 7.

Another matter concerns the shape of the bee's eye. Our measurements relate only to a uni-dimensional resolution on the eye. The frequency curves in Figs. 7 and 8 therefore concern the distribution of functional elements in one dimension only. It might then seem that, though useful as description, such curves cannot represent any-

thing basic in the constitution of the ocular mosaic. Since the elements in the eye end in a surface, it might appear that a frequency distribution per unit area would be more fundamental than a distribution per unit distance. The simplest method of converting a linear distribution into a surface distribution would be, in the case of a uniform mosaic, to consider the square of the linear distribution.

There are however several reasons which render it probable that a linear frequency distribution is nearer reality than a surface one. The eye is about four times as long as it is wide. Furthermore the angular separation between adjacent ommatidia is more than three times as great in the horizontal meridian as in the vertical meridian. Both these facts would tend to make the bee's eye an organ which functions essentially as a linear receptor.

This is indeed true experimentally. Baumgärtner found that bees in the field are very astigmatic, and resolve their environment vertically with much greater accuracy than horizontally. Our own experiments showed this independently. Our first tests were made with a long creeping compartment in which the bee crawled from one end to the other, and we tried to make it slow up or turn around by moving a set of stripes under it either in the direction in which the bee was creeping or in the opposite direction. We got no reliable results this way. It was only when the apparatus was changed so that the bee crawled in a direction perpendicular to the movement that we secured significant results. The bee's eye thus seems to be functionally and structurally an organ concerned with the resolution of the environment mainly in one dimension. The data, and the statistical curves in Figs. 7 and 8, therefore very likely represent the real rather than an apparent distribution of elements in the ocular mosaic.

SUMMARY.

1. Bees respond by a characteristic reflex to a movement in their visual field. By confining the field to a series of parallel dark and luminous bars it is possible to determine the size of bar to which the bees respond under different conditions and in this way to measure the resolving power or visual acuity of the eye. The maximum visual acuity of the bee is lower than the lowest human visual acuity. Un-

der similar, maximal conditions the fineness of resolution of the human eye is about 100 times that of the bee.

2. The eye of the bee is a mosaic composed of hexagonal pyramids of variable apical angle. The size of this angle determines the angular separation between adjacent ommatidia and therefore sets the structural limits to the resolving power of the eye. It is found that the visual angle corresponding to the maximum visual acuity as found experimentally is identical with the structural angular separation of adjacent ommatidia in the region of maximum density of ommatidia population. When this region of maximum ommatidia population is rendered non-functional by being covered with an opaque paint, the maximum visual acuity then corresponds to the angular separation of those remaining ommatidia which now constitute the maximum density of population.

3. The angular separation of adjacent ommatidia is much smaller in the vertical (dorso-ventral) axis than in the horizontal (anterio-posterior) axis. The experimentally found visual acuity varies correspondingly. From this and other experiments as well as from the shape of the eye itself, it is shown that the bee's eye is essentially an instrument for uni-directional visual resolution, functional along the dorso-ventral axis. The resolution of the visual pattern is therefore determined by the vertical angular separation of those ocular elements situated in the region of maximum density of ommatidia population.

4. The visual acuity of the bee varies with the illumination in much the same way that it does for the human eye. It is low at low illuminations; as the intensity of illumination increases it increases at first slowly and then rapidly; and finally at high intensities it becomes constant. The resolving power of a structure like the bee's eye depends on the distance which separates the discrete receiving elements. The data then mean that at low illuminations the distance between receiving elements is large and that this distance decreases as the illumination increases. Since such a moving system cannot be true anatomically it must be interpreted functionally. It is therefore proposed that the threshold of the various ommatidia are not the same but that they vary as any other characteristic of a population. The visual acuity will then depend on the distance apart of those elements

whose thresholds are such that they are functional at the particular illumination under investigation. Taking due consideration of the angular separation of ommatidia it is possible to derive a distribution curve for the thresholds of the ommatidia which resembles the usual probability curves, and which describes the data with complete fidelity.

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THE CONCENTRATION EFFECT IN NITELLA.*

By W. J. V. OSTERHOUT and E. S. HARRIS.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Different concentrations of the same salt brought in contact with living tissue commonly show a P.D. which is known as the concentration effect. Since it can exist only when the surface is permeable to ions[1] it is obviously important for the study of permeability provided we can distinguish between effects due to the living protoplasm and those due to cell walls or other non-living structures. This viewpoint occasioned the present investigation. It early became apparent that in order to interpret the results a picture of conditions in the cell must be worked out which has necessitated additional experimentation.

Quantitative studies of concentration effects were first made by MacDonald,[2] experimenting on nerves but without distinguishing carefully between living and dead cells. The theoretical implications of such measurements were first pointed out by Loeb and Beutner.[3] They investigated plant organs with a cuticularized cell wall which may have been largely responsible for the observed effects;[4] their experiments on muscle,[5] like those of Matsuo[6] on frog liver, gave very little con-

* The authors desire to express their gratitude to the Carnegie Institution of Washington, D. C., which generously provided for the beginning of these investigations (1922–25).

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1927–28, xi, 83.

[2] MacDonald, J. S., *Proc. Roy. Soc. London*, 1900, lxvii, 310; *Thompson Yates Laboratory, Liverpool*, 1902, iv, pt. 2.

[3] Loeb, J., and Beutner, R., *Biochem. Z.*, 1912, xli, 1; xliv, 303; 1913, li, 300. *Cf.* Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920.

[4] Beutner[3] states that the same results were obtained after the tissue was killed by chloroform. See also Fujita, A., *Biochem. Z.*, 1925, clviii, 11.

[5] The results of Loeb and Beutner on the finger nail and the skin are probably due in large part to non-living elements.

[6] Matsuo, T., *Arch. ges. Physiol.*, 1923, cc, 132.

centration effect. Recent experiments by Jost[7] have dealt with the concentration effect in *Nitella* without separating the effect due to the protoplasm from that of the cell wall: but in the experiments of Brooks[8] the effect of the cell wall seems to have been avoided.

Our experiments on *Nitella* have been carried on at various times from 1922 onward. The range of concentrations studied was as a rule from 0.001 M to 0.1 M. Above this plasmolysis and injury are apt to occur and in very dilute solutions there is danger of contamination. The experiments were arranged as in Fig. 1. The temperature averaged about 23°C. Details of technique are given in previous papers.[9]

In all cases the results were recorded photographically and the measurements were made from these records.

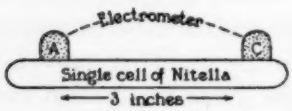

FIG. 1.

FIG. 1. Diagram to show the arrangement of the experiments. Flowing junctions or wads of cotton soaked in solutions are applied at *A* and *C* or the ends are allowed to dip into cups containing solutions. Cotton soaked in distilled water is in some cases applied for a short stretch between *A* and *C* but a space is left on each side of the cotton to prevent short-circuiting.

It was first necessary to ascertain whether the observed concentration effect is entirely due to the protoplasm or whether the cell wall also plays a part.

In order to study the cell wall dead cells were emptied of sap by cutting off one end and squeezing out their contents. They were then allowed to fill with tap water, and again squeezed out and allowed to fill with tap water. This was repeated several times. Their contents were then squeezed out and they were then filled with air and used at once. In this way it is believed that we may approximate the condition of the cell wall in the living cell where there is probably not much ionic exchange between the cell wall and the protoplasm.

The ends of the dead cell walls (filled with air) were allowed to dip into solutions (in which calomel electrodes were immersed) in such fashion that the solutions did not creep into the lumen (if this occurs it may cause variations in the magnitude or even in the sign of the P.D.). On account of the high resistance the meas-

[7] Jost, L., *Sitzungsber. Heidelberger Akad. Wissensch., Abt. B*, 1927, Abhandl. 13, Nov.

[8] Brooks, S. C., and Gelfan, S., *Protoplasma*, 1928, v, 86. (This article was received after the completion of this paper.)

[9] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, (*a*) 1927–28, xi, 673; (*b*) 1928–29, xii, 167.

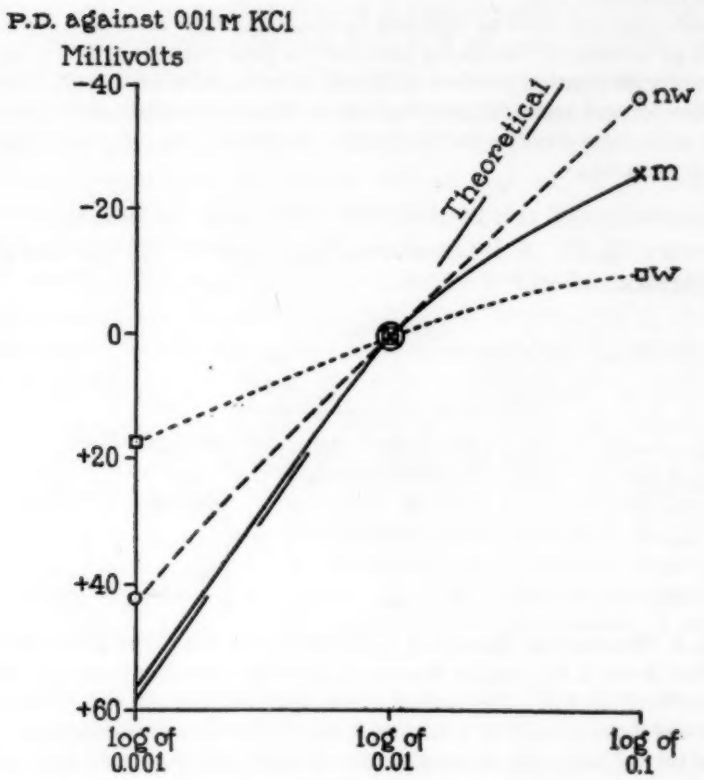

FIG. 2. Curves showing the concentration effect of KCl (each point is the average of 10 to 130 determinations, the probable error of the mean being in general less than 10 per cent of the mean). Curve *m*, concentration effect of the protoplasm alone (*i.e.*, on the *m* basis); Curve *nw*, that of the cell wall and protoplasm combined (*i.e.*, on the *nw* basis); Curve *w*, that of the cell wall alone. The potential difference across the protoplasm when in contact with 0.01 M KCl is taken as zero for purposes of comparison (thus if the curve shows 60 millivolts at 0.001 M KCl it means that this is the potential difference between 0.001 M KCl and 0.01 M KCl). The actual value (called the *AP* value) across the protoplasm in contact with 0.01 M KCl is −2.9 millivolts (average of 60 experiments).

For Curve *m* the values are +56.7 and −25.4; for Curve *nw* +42.2 and −37.9; for Curve *w* +16.8 and −10.

urements on the dead cell wall were made with a Compton electrometer (Cambridge Instrument Co.).

Flowing contacts must be used with caution (especially when the solutions are dilute) on account of "streaming potential" (a good criterion of this is the P.D. observed when identical solutions are placed at the opposite ends, one flowing and the other not, and unless this gave approximately zero the experiment was rejected: in all cases these results were checked by the employment of cups instead of flowing contacts).

Experiments of this kind on cell walls gave the results shown in Curve *w* (Fig. 2). It is therefore evident that the cell wall can give a concentration effect.[10]

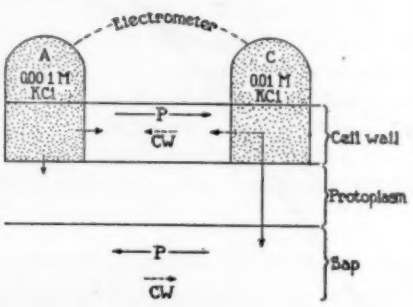

FIG. 3. Hypothetical diagram of conditions in the cell. The arrows show the direction in which the positive current tends to flow and their length the relative magnitude of the P.D. The vertical arrows represent the P.D. due to the protoplasm and the resulting P.D. is labelled *P*; the broken arrows represent the E.M.F. due to the cell wall, and the resulting P.D. labelled *CW*, is opposed to *P*. These relations are more simply represented in Fig. 4.

In order to have some picture of the conditions possibly existing in the cell we may perhaps employ the diagram in Fig. 3 (without by any means assuming that it is correct in all respects). The arrows show the direction in which the positive current tends to flow and their lengths the relative P.D. The vertical arrow at *C* represents the P.D.

[10] In *Nitella* the cell wall is much less cuticularized than the epidermis of the plants used by Loeb and Beutner and it gives a much lower concentration effect.

It may be added that when cells are killed by being placed for 10 minutes in pure chloroform (not in an aqueous solution), removed, and placed in the apparatus they may give a higher concentration effect than the dead cell wall treated as described above.

across the protoplasm: this is opposed by the vertical arrow at A. The arrow at C is longer than that at A since the higher concentration is at C (this would be the case theoretically if the cation has the greater tendency to enter or the greater mobility in the protoplasm) which is in accord with all our measurements.[9a] The resultant P.D. due to the protoplasm is shown by the arrow labelled P (*i.e.*, the current tends to flow from C through the protoplasm and sap to A and thence through the cell wall to C). (We neglect for the present any short circuit through the protoplasm.)

The relations are represented more simply in Fig. 4 where the resultant resistance of the protoplasm and sap is represented by R_P and that of the cell wall by R_O. The P.D. in the protoplasm is represented by E_{PA} and E_{PC}.

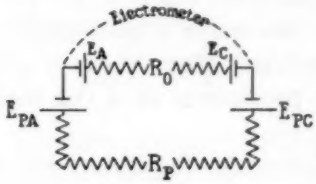

FIG. 4.

FIG. 4. As in Fig. 3 except that the resultant resistance of the protoplasm and sap is represented by R_P (with one horizontal and two vertical components) and that of the cell wall by R_O (having only a horizontal component). The P.D. of the protoplasm is represented by E_{PA} and E_{PC}, that of the cell wall by E_A and E_C.

The relations in the cell wall may be inferred from the fact that when a strip of dead cell wall is used we find a positive current flowing through the electrometer from the dilute to the concentrated solution (which should be the case theoretically if the cation has a greater tendency to enter or a greater mobility than the anion). The P.D. is represented by arrows parallel to the surface (broken arrows) because the applied solution quickly penetrates through the wall (in a few seconds[11]) and then begins to diffuse along the wall, setting up the P.D. shown by the broken arrows in the figure: the resultant P.D. due to the cell wall is labelled CW and is opposite to that labelled P.

It is therefore evident that the concentration effect of the cell wall

[11] This is shown by the fact that substituting 0.01 M NaCl for 0.01 M KCl has practically no effect on the dead cell so that when such a change is made on the living cell we must regard the effect as due entirely to the protoplasm: the chief electrical disturbance due to such a change lasts only a few seconds during which time the solution must have penetrated through the wall to the protoplasm.

tends to increase the values due to the protoplasm. For example, in Fig. 4 (where the broken arrow at C is represented by E_C and that at A by E_A) we may write[12]

$$\text{Obs. conc. effect} = (E_{PC} - E_{PA}) \frac{R_O}{R_O + R_P} + (E_C - E_A) \frac{R_P}{R_O + R_P}$$

Here $E_{PC} - E_{PA}$ corresponds to the arrow labelled P in Fig. 3 and $E_C - E_A$ to that labelled CW. Since R_P is small[9a] compared to R_O the effect of the cell wall is probably small.

In order to eliminate the action of the cell wall and to ascertain the concentration effect due to the living protoplasm we may apply identical solutions to A and C, and kill C.[13] If, for example, we lead off at A and C with 0.01 M KCl we eliminate the effect of the cell wall but this gives no P.D. until we kill C (which reduces the E.M.F. of the protoplasm at C approximately to zero[14]). We make a photographic record on which we measure the P.D. immediately after the death of C to find the potential difference across the protoplasm at A (we shall call this the AP value).

In order to see what this means let us consider the factors which determine the P.D. We may assume that under normal conditions all the E.M.F. at A is balanced by corresponding E.M.F. at C so that if we lead off from A to C with the same solution the P.D. will be zero. When we change the salt solution at A it seems probable that its penetration is so slow that in our very brief experiments we make no change in the protoplasm except at the outer surface of X.

The electromotive force at the outer surface of X may be called E_{AX}, the corresponding E.M.F. at C being E_{CX} (the E.M.F. in the region lying between A and C, or outside these points, may be neglected for our present purpose). If the E.M.F. at the outer surface of X

[12] It is assumed that the resistance of the electrometer is sufficiently high to preclude any error in measurement and this is the case even when a grid leak is used to shunt the grid and filament (the resistance of the grid leak is 20 megohms).

[13] As shown in a former paper[9a] killing C usually produces no immediate effect at A, which can be detected by our present methods.

[14] The killing of C apparently produces no effect on the cell wall. There is, of course, some current flowing in the cell wall but the experiments of Dr. Blinks indicate that this produces no polarization.

in contact with 0.01 M KCl be called $E_{AX\ 0.01\ KCl}$ and all other E.M.F. at A be designated as E_{AZ} the total E.M.F. at A is $E_{AX\ 0.01\ KCl} + E_{AZ}$. What we measure is probably a certain fraction of this which we will call m,[15] so that the observed P.D. across the protoplasm at A is

$$\text{Observed P.D.} = m(E_{AX\ 0.01\ KCl} + E_{AZ}) = mE_{AX\ 0.01\ KCl} + mE_{AZ}$$

(The E.M.F. in the cell wall at A and C balances out since both spots are in contact with the same solution.[16])

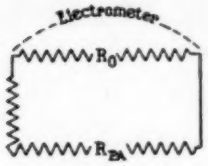

FIG. 5. Hypothetical diagram of electrical resistances in the protoplasm when C is killed. R_O (with horizontal component only) represents the resistance of the cell wall and R_{PA} (with one vertical and one horizontal component) that of the protoplasm and sap.

FIG. 5.

When we substitute 0.001 M KCl for 0.01 M KCl at A and C we change $E_{AX\ 0.01\ KCl}$ to $E_{AX\ 0.001\ KCl}$ but in brief experiments we may assume that E_{AZ} will not change. If we subtract the observed P.D. across the protoplasm in contact with 0.01 M KCl from that across the protoplasm in contact with 0.001 M KCl we have

$$\text{Observed concentration effect} = mE_{AX\ 0.001\ KCl} - mE_{AX\ 0.01\ KCl}$$

The value of m depends on the resistance of the cell wall (R_O) between A and C and the resultant resistance (R_{PA}) of the protoplasm and sap in the circuit after C is killed. If we represent the resistances as shown in Fig. 5 we are measuring the P.D. across R_O and the value

[15] This has been discussed in a previous paper[9a] (where n was used in place of m).

[16] This would not be true if we did not employ a flowing contact at C for sap would come out through the dead protoplasm and change the concentration in the wall, unless the applied solution were sap or 0.05 M KCl or its equivalent. But the flowing contact appears to prevent any disturbance due to the coming out of sap as is shown in a former paper.[9a] Care must be taken to avoid any disturbances due to "flowing potential."

of m will therefore be $m = R_O \div (R_O + R_{PA})$. Measurements[17] made by Dr. Blinks indicate that when C is killed and the solution at A is 0.001 M KCl (or a higher concentration), with the cell wall between A and C imbibed with tap water, the values of R_O and R_{PA} are such that m is between 0.8 and 1.0.

In order to get some idea of the change in resistance due to killing C we apply 0.01 M NaCl to A and 0.01 M KCl to C (without killing A

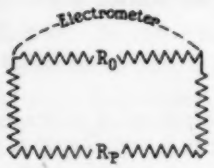

FIG. 6. As in Fig. 5 but with both ends intact (R_P in place of R_{PA}).

FIG. 6.

or C). Since $E_{CX\ 0.01\ KCL} = E_{AX\ 0.01\ KCL}$ the observed effect (which will be called the chemical effect) may be regarded as

$$\text{Obs. chem. effect} = nE_{AX\ 0.01\ NaCl} - nE_{CX\ 0.01\ KCl} = nE_{AX\ 0.01\ NaCl} - nE_{AX\ 0.01\ KCl}$$

where $n = R_O \div (R_O + R_P)$ and R_P represents the resultant resistance of the protoplasm and sap in the circuit in the intact cell as indicated in Fig. 6. (The P.D. of the cell wall may be neglected since the cell wall alone gives no chemical effect.)

If we now kill C and ascertain the chemical effect (in the same manner as for the concentration effect, *i.e.*, by measuring the AP value at A of 0.01 M NaCl and then that of 0.01 M KCl) we shall have two measurements, *i.e.*, $m(E_{AX\ 0.01\ NaCl} - E_{AX\ 0.01\ KCl})$ and $n(E_{AX\ 0.01\ NaCl} - E_{AX\ 0.01\ KCl})$. From these measurements we find that the value of $m \div n$ is not far from 1 and direct measurements of resistance made by Dr. Blinks indicate that the value of n is not much

[17] We regard R_{PA} and R_O as resistances in parallel whose resultant resistance is equal to $\dfrac{(R_{PA})\,(R_O)}{R_{PA} + R_O}$ which is measured by determining the resistance of the circuit through A and C after C has been killed. We then measure an equal length of cell wall blown up with air (after the protoplasm has been squeezed out) to obtain R_O. From this we obtain R_{PA}.

below that of m. In other words we compare the values of $m = \frac{R_O}{R_O + R_{PA}}$ and $n = \frac{R_O}{R_O + R_P}$ and find that the difference between R_P (the resistance of the protoplasm and sap) and R_{PA} (the same resistance after killing C) is not enough to have any great effect on the values of m and n: this is, of course, due to the very high value of R_O (according to the measurements of Dr. Blinks the resistance of the cell wall, imbibed with tap water, is considerably over 4 megohms per inch).

These results are checked by applying 0.001 M KCl at A and the same saturated with chloroform at B (Fig. 7): at the same time we

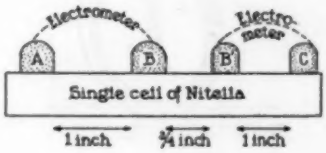

FIG. 7. Diagram to show arrangement of experiments.

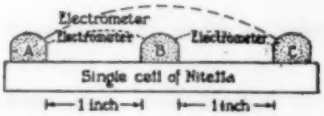

FIG. 8. Diagram to show arrangement of experiments.

apply 0.01 M KCl at B' and the same saturated with chloroform at C (Fig. 7). We thus measure simultaneously the AP value of the two concentrations and by subtracting one from the other obtain the concentration effect. In this case we eliminate both the cell wall effect and errors due to the use of different cells but even so there may be considerable variation. The results agree with those previously described.

Still another method is to arrange the experiments as in Fig. 8 and to kill C with 0.01 M KCl saturated with chloroform. We apply 0.01 M KCl at B and 0.001 M KCl at A. Measuring from A to B gives us the concentration effect of the protoplasm on the n basis together with the cell wall effect: we may call this the nw basis. We

may get the *m* basis by measuring from *B* to *C* and subsequently replacing the 0.01 M KCl at *B* and *C* with 0.001 M KCl. We are thus able to measure a single cell on the *m* and on the *nw* basis and this may also be done for the chemical effect (*n* basis) to compare the values of *m* and *n*. Here too the results are similar to those already described.

It may be added that when the cell wall is imbibed with 0.001 M KCl and we place 0.01 M KCl at *C* and 0.001 M KCl at *A* the results

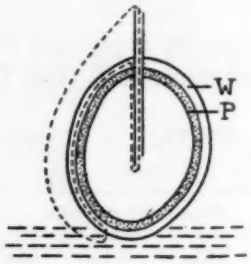

FIG. 9. Diagram to show the electrical leak when a capillary is inserted into *Valonia*.

are practically the same as when the cell wall is imbibed with tap water.

The matter may be approached from another standpoint, *i.e.*, by inserting a capillary. It has been shown in a former paper[18] that when a capillary is inserted into *Valonia* (Fig. 9) we may have a

[18] Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927–28, xi, 193. The use of the capillary in *Valonia* was begun by one of us in the summer of 1923 (for the first reference to this see Osterhout, W. J. V., *J. Gen. Physiol.*, 1924–25, vii, 561). In the summer of 1925 Taylor and Whitaker (Taylor, C. V., and Whitaker, D. M., Carnegie Institution Year Book, 1925–26, No. 25, p. 248) introduced a microelectrode and recently Jost (Jost, L., *Sitzungsber. Heidelberger Akad. Wissensch.*, 1927, Abteilung 13, Nov.) has employed a capillary.

The use of the capillary in *Nitella* was begun by us in 1922 (cf. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 391). Previous to this Miss Nichols (Nichols, S. P., *Bull. Torrey Bot. Club*, 1925, lii, 351) introduced needles to observe the effect upon structure and upon streaming. Recently Taylor and Whitaker (Taylor, C. V., and Whitaker, D. M., *Protoplasma*, 1927, iii, 1) and Brooks and Gelfan[8] have employed a microelectrode.

leak at the point of insertion but if we wait for a seal to form this leak disappears and we then may get the true value of the P.D. across the protoplasm. In the case of *Nitella* it is not necessary to wait for a seal to form since the resistance of the cell wall imbibed with tap water is so high that the leak is not important. Hence we may approximate the true values by measuring as soon as the capillary is inserted.

For this purpose the cell is placed on the paraffin block on the stage of a microscope and the capillary (filled with artificial sap) is held firmly in place near the end of the cell. We then seize with forceps a strip of dead cell wall adhering to the

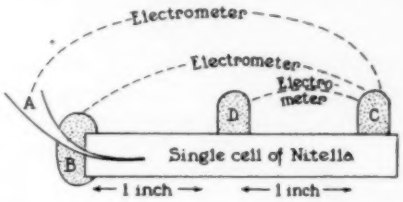

FIG. 10. Diagram to show the arrangement of the experiment when a capillary (*A*) is inserted in the cell.

end of the living cell (this strip is a part of the neighboring cell whose end has been cut away) and pull the living cell along until it impales itself on the capillary. One can then observe with the microscope whether the capillary is projecting far enough into the vacuole to indicate that its point is not covered by a layer of protoplasm.

An experiment was arranged as shown in Fig. 10. By means of a rotary switch[19] we recorded the circuit from the interior of the capillary *A* to *C* (Fig. 11, Curve *A*, long dash): from the outside of the capillary where it pierced the cell wall, *B*, to *C* (Curve *B*, medium dash) and from *D* to *C* (Curve *D*, short dash). If such an experiment were performed on *Valonia* (Fig. 9) there would be a good deal of difference between *A* and *B* provided we had allowed the cell to form an electrical seal but in the case of *Nitella* no such seal is formed as the cell is used immediately after the insertion of the capillary and in consequence there is little difference between *A* and *B*.

[19] This was described in a previous paper (Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 391).

The values observed at *A* and *B* (Fig. 11) are a measure of the P.D. across the protoplasm at *C* (*AP* value) as would be the case if we killed with chloroform at *B*. (This *AP* value is negative but appears positive on the record because curves *A* and *B* record the inside of *C* instead of the outside.) The fact that *A* and *B* differ somewhat might be taken to indicate that the point of the capillary has gone through the protoplasm into the vacuole. If it had merely indented the protoplasm, making a deep pocket, without actually piercing it we might expect *A* and *B* to be identical.

The short dash, Curve *D*, records the P.D. of *D* against *C* (which is about 1 millivolt negative); the fact that there is so little differ-

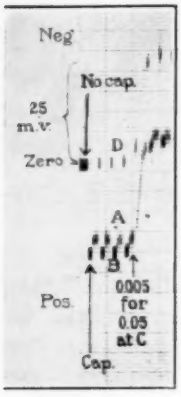

FIG. 11. Photographic record showing potential differences. The experiment is arranged as shown in Fig. 10 with 0.05 M KCl at *B*, *D*, and *C*. Before the capillary is inserted there is only one curve (marked "No cap."). As soon as the capillary is inserted the record shows three curves: Curve *A* records the potential difference between the capillary and *C*; Curve *B* that between *B* and *C*; and Curve *D* that between *D* and *C*. When 0.005 M KCl is substituted for 0.05 M KCl at *C* the curves rise together. The vertical marks represent 5-second intervals.

FIG. 11.

ence between *D* and *C* indicates that injury has not spread from the point of entrance of the capillary to *D* (for when *D* is dead its apparent P.D. will be the same as that of the capillary).

When 0.005 M KCl is substituted for 0.05 M KCl at *C* both curves rise simultaneously (since *C* becomes more positive which makes both the capillary and *B* appear more negative) to a degree which indicates that *C* is not injured. The amount of this rise is the observed concentration effect which is measured on the equivalent of the *m* basis from *A* to *C* and on the *nw* basis from *D* to *C*. There is evidently no great difference between the two and this might be taken to indicate that the cell wall effect does not greatly alter the value due to the

protoplasm alone. For when we change from 0.05 to 0.005 M KCl at *C* we presumably have a cell wall effect when we measure from *D* to *C* but not when we measure from *A* to *C* or from *B* to *C*, yet the measurement (*i.e.*, the amount of rise) is practically the same in all cases.

Let us now consider certain other aspects of the experiments on *Nitella*. It might be thought that killing *C* tends to alter the concentration effect at *A* (as would be expected if *A* were injured). Hence experiments were made on intact cells having unlike concentrations at *A* and *C* (*i.e.*, on the *nw* basis). The average measurements of 130 such cells (Fig. 2, Curve *nw*) do not agree closely with those made on the *m* basis (58 cells, Fig. 2, Curve *m*), but this lack of agreement seems to have no theoretical significance. On the basis of Figs. 3 and 4 we might expect the values on the *m* basis to be approximately equal to those on the *nw* basis minus the cell wall effect. While this may be approximately true for the value of 0.01 *vs.* 0.1 M KCl it is just the reverse for the value of 0.01 *vs.* 0.001 M KCl. In view of this we are not inclined to attribute much quantitative significance to any of these figures.[20]

Experiments in which *C* was killed (with 0.01 M KCl plus chloroform) and kept in contact with 0.01 M KCl while the solution in contact with *A* was varied (which may be called the *mw* basis, since it includes the effect of the cell wall) gave results much like those in Curve *m*, indicating that the cell wall effect does not greatly affect the results (*i.e.*, curves on the *m* basis do not differ greatly from those on the *mw* basis).

The curves in Fig. 2 are concave to the axis of the abscissæ. Somewhat the same sort of curve was found by Beutner[3] in studying organic substances immiscible with water,[21] although theoretical considerations (to be discussed a little later) lead us to expect in the ideal case the straight line labelled "theoretical" in Fig. 2.

[20] We do not know to what extent the values are cut down by short circuits and eddy currents: for example, the current due to the broken arrow at *C*, Fig. 3, might travel in the cell wall only a short distance toward *A* before passing into the protoplasm.

[21] This is discussed by Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, 200.

In addition to the results shown in Fig. 2 there are a few anomalous cases which should be mentioned. Under certain circumstances the cell may produce a high P.D. (up to 120 millivolts) even with identical solutions at A and C. It is not surprising therefore that in certain kinds of material we find a concentration effect of 100 millivolts or more for a tenfold dilution (*e.g.*, 0.001 M *vs.* 0.01 M KCl). Since the theoretical limit for a tenfold dilution is about 60 millivolts it is natural to assume that in these anomalous cases the extra P.D. comes from the deeper layers, Y or W.

In view of such results as those of Michaelis and Fujita[22] on gelatin and of Amberson and Klein[23] on dead frog skin, which show a change of sign of the concentration effect with alteration of pH value, it may be well to state that no such change is found in living cells of *Nitella* if in the range studied (pH 5 to 9.5) the concentration of H^+ is small in comparison to that of other cations.

In view of the fact that organic substances immiscible with water containing organic acids show a concentration effect with KCl with the dilute solution positive while others containing alkalies show the dilute solution to be negative, it may be well to state that somewhat similar results may be obtained by means of a string soaked in acid or alkali, the result being easily accounted for as diffusion potential.

Let us now consider the interpretation or our results. If we regard the P.D. as due primarily to diffusion potential we shall conclude that the mobility of K^+ in the protoplasmic surface is greater than that of Cl^-. But if it is due to phase boundary potentials we may employ the usual formula, putting $R' = \dfrac{R}{0.4343\ n\ F}$ and $n = 1$

$$\text{P.D.} = R'T \log \left(\frac{C_{coh}}{C_{czh}}\right)\left(\frac{C_{czl}}{C_{col}}\right)$$

where C_{czh} and C_{czl} are the concentrations of the cations in X at the high and low concentrations respectively and C_{coh} and C_{col} are the corresponding concentrations in the external solution. The sign is that of the dilute solution.

[22] Michaelis, L., and Fujita, A., *Biochem. Z.*, 1925, clxii, 245.

[23] Amberson, W. R., and Klein, H., *J. Gen. Physiol.*, 1927–28, xi, 823.

If $C_{cxh} = C_{cxl}$ we have

$$\text{P.D.} = R'T \log \frac{C_{coh}}{C_{col}}$$

In metallic electrodes (and in some cases in glass electrodes[24]) this equation applies but it is a question to what extent this is true of protoplasm.[24]

In order to picture the relations involved it may be convenient to employ the treatment of Nernst, as discussed in a former paper,[1] where it is stated that if, for example, we apply equimolar solutions of RbCl and CsCl we may write

$$\text{P.D.} = \frac{R'T}{2} \log \frac{A_{Cs}}{A_{Rb}}$$

where A_{Cs} and A_{Rb} are the "true"[25] partition coefficients of Cs and Rb. The sign is that of the solution of Rb as shown by the electrometer. If we use two salts without a common ion, *e.g.*, equimolar solutions of RbCl and CsBr, we may take first the phase boundary potential at the spot in contact with RbCl: this is[26]

$$\text{P.D.} = \frac{R'T}{2} \log \frac{A_{Cl}}{A_{Rb}}$$

the positive sign meaning that the positive current tends to flow from Y to X in the protoplasm.[27] The corresponding value for CsBr would be

$$\text{P.D.} = \frac{R'T}{2} \log \frac{A_{Br}}{A_{Cs}}$$

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[24] This has been discussed in a former paper.[1]

[25] The true partition coefficient of Rb is the partition coefficient (in this case the concentration at equilibrium in the protoplasmic layer X divided by the concentration in the external solution) it would have if it could enter unhindered by Cl, or in other words if Rb and Cl had the same "true" partition coefficient.

[26] For a discussion of this see[1].

[27] This is adopted as a convention.

Subtracting one from the other we have

$$\text{P.D.} = \frac{R'T}{2} \log \left(\frac{A_{\text{Cl}}}{A_{\text{Rb}}}\right)\left(\frac{A_{\text{Cs}}}{A_{\text{Br}}}\right)$$

The sign is that of the solution of RbCl, as shown by the electrometer.

We may calculate the concentration effect in similar fashion. If the "true" partition coefficient of the cation is A_{cl} at the low concentration and A_{ch} at the high concentration (the corresponding symbols for the anion being A_{al} and A_{ah}) we may for purposes of illustration make the following substitutions in the last equation: $A_{cl} = A_{\text{Rb}}$, $A_{ch} = A_{\text{Cs}}$, $A_{al} = A_{\text{Cl}}$, and $A_{ah} = A_{\text{Br}}$. We then have

$$\text{P.D.} = \frac{R'T}{2} \log \left(\frac{A_{al}}{A_{cl}}\right)\left(\frac{A_{ch}}{A_{ah}}\right)$$

The sign is that of the dilute solution as shown by the electrometer. It is evident from this equation that in order to get a concentration effect there must be a difference between the "true" partition coefficients of either the cations or of the anions at the two concentrations.

It may be of interest to see how variation of "true" partition coefficients will affect the sign of the dilute solution. If A_c and A_a, as well as $A_c \div A_a$, increase as the concentration increases the tendency of the cations to pass into X will always be greater at the higher concentration so that the positive current will tend to flow from the lower concentration through the electrometer to the higher (dilute solution positive) and at the same time relatively more of the salt will enter at the higher concentration. This would correspond to those cases described by Wosnessensky[28] in experiments on amyl alcohol (*e.g.*, the concentration effect of LiCl) where the dilute solution is positive.[29]

[28] Wosnessensky, S., *Z. physik. Chem.*, 1925, cxv, 405; cxvii, 457. Wosnessensky, S., and Astachow, K., *Z. physik. Chem.*, 1925, cxviii, 295; 1927, cxxviii, 362. Wosnessensky, S., Astachow, K., and Tschmutow, K., *Z. physik. Chem.*, 1926, cxxi, 143.

[29] In comparing two salts it must be borne in mind that if one is taken up more than the other it does not necessarily mean that in the former case there are more ions in X since the dissociation constants in X may differ. In our discussion we shall assume that the dissociation constants are not very different.

But if $A_c \div A_a$ and A_c increase with concentration while A_a decreases as the concentration increases the dilute solution will still be positive although relatively less salt will be taken up at the higher concentration and this may be true even if both A_c and A_a decrease as the concentration increases.

If on the other hand $A_c \div A_a$ decreases as the concentration increases the dilute solution will be negative even though A_a increases as the concentration increases or though both A_c and A_a increase as the concentration increases (in the latter case the penetration of salt increases as the concentration increases).

We therefore see that on this basis the sign of the dilute solution depends on the behavior of $A_c \div A_a$ and not on the relative amount of salt taken up.

According to recent researches[30] and theoretical discussions[31] little or no P.D. is to be expected with KCl at phase boundaries. On the other hand Michaelis and others[32] find fairly high values for the P.D. of KCl with collodion membranes. This might lead to the suspicion that the results found with *Nitella* are largely due to the cell wall but it is evident from what has been said that this is not the case. The concentration effect of the protoplasm is much greater than that of the cell wall and this difference is much more striking in the case of the chemical effect. It should be noted that collodion membranes give the same sort of chemical effect with KCl and NaCl as does protoplasm.

There are difficulties in regarding the P.D. as due to Donnan effect, since the cell cannot very well be in Donnan equilibrium with two different concentrations at the same time. We must assume that the indiffusible ion is positive in order to make the dilute solution positive. The numerical relations may be illustrated as follows: If we have inside an indiffusible anion $R^- = 21$ and outside KCl = 10 we have $(K^+_{outside})\ (Cl^-_{outside}) = 100$: inside the product of K^+ (= 25) and

[30] *Cf.* Holleman, L. W. J., and Werre, J. P., *Rec. trav. chim. Pays-Bas*, 1928, ser. 4, xlvii, 105.

[31] *Cf.* Cremer, M., in Bethe, A., *et al.*, Handbuch der normalen und pathologischen Physiologie, Berlin 1928, viii, 1034.

[32] *Cf.* Michaelis, L., and others. Various articles in *J. Gen. Physiol.*, 1925–29, ix–xii.

Cl^- (= 4) is also 100. If we now raise the outside concentration to 100 we have outside (K^+) (Cl^-) = 10,000 and inside the product of K^+ (= 111.1) and Cl^- (= 90.1) is also 10,000 (R^- being 21 as before). In the former case the value of $\frac{C_{co}}{C_{ci}}$ is $\frac{10}{25}$ and in the latter $\frac{100}{111.1}$. We then employ the usual formula

$$\text{P.D.} = R'T \log \frac{C_{co}}{C_{ci}}$$

(where C_{co} is the concentration of cations outside and C_{ci} the concentration inside) which would give for the lower concentration P.D. $= R'T \log \frac{25}{10}$ and for the higher concentration P.D. $= R'T \log \frac{111.1}{100}$ so that the concentration effect would be

$$\text{P.D.} = R'T \log \left(\frac{25}{10} \div \frac{111.1}{100} \right) = R'T \log 2.25$$

The dilute solution would be positive but the P.D. would be less than with a metallic electrode where the concentration effect would be $R'T$ log 10 (which at 18°C. would be 58 millivolts). Hence the assumption of a Donnan equilibrium as the explanation of the P.D. may not give values as high as occur in dilute solutions in the case of living cells (whose values approximate those found with metallic electrodes). In order to get values approaching those for metallic electrodes we should have to assume values for R^- more than 10 times as great as those given above.

It is evident that as the more dilute solution of KCl is positive with living protoplasm K^+ tends to enter and the value of $A_c \div A_s$ must increase with the concentration or else the mobility of K in the protoplasm must be greater than that of Cl^-.

There is no direct proof that Cl^- tends to penetrate[33] in ionic form.

Let us now consider the bearing of our results upon electrical conditions in the protoplasm. Adopting the hypotheses set forth in a former paper[1, 9a] that the protoplasm consists of layers with different

[33] It may reach the vacuole by passing through the non-aqueous layers of the protoplasm in the undissociated condition.

properties we might diagram the electrical conditions as shown in Fig. 12. The X and Y arrows marked A and C under "Net result" at the left have had their directions determined by considerations set forth in a former paper;[9a] and this is confirmed by the fact that when sap is applied to the outer surface of X (as at the right) the result shows that the X arrow (X_c) is longer than the Y arrow (Y_c).[9a] This might be regarded as indicating that X gives a greater P.D. with sap than does Y in which case we might make the arrow at the external surface of X longer than that at the inner surface of Y, as is done in the figure: we should in that case be inclined to suppose that X gives a greater P.D. against W than does Y and to make the arrows at the outer and inner surfaces of W of corresponding length (as in the figure). The arrows at b, c, and d will be of the same length at A as at C no matter what the external solutions may be, since in

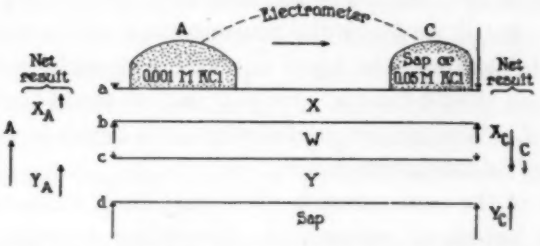

FIG. 12. Hypothetical diagram to show the condition of the protoplasm in contact with different concentrations of KCl. The direction of the arrows indicates how the positive current tends to flow and their length the relative P.D.

brief experiments such as we perform the arrow at a is the only one to change, except in case of injury.

If the electrolyte in W should happen to be mostly KCl the fact that with the protoplasm in contact with 0.001 M KCl the arrow at a is shorter than that at b would mean that the concentration of KCl in W was greater than 0.001 M. But if the electromotive forces at b and c are chiefly due to the diffusion potential, for example of an organic substance produced in the protoplasm, we cannot draw any conclusions regarding the concentration of inorganic salts in W.

If we suppose that X and Y are non-aqueous layers and that W is aqueous we may surmise that each surface is the seat of electromotive

forces as shown in Fig. 12. When we apply 0.001 M KCl at A and 0.1 M KCl at C it is probable that under normal conditions the E.M.F. at A and C is equal and opposite except those at the outer surface which are represented in the diagram by arrows of unequal length. If we regard the observed effects as due to diffusion potentials we should say that the cations appear to have a greater mobility in X than the anions and consequently we should expect the arrows at the outer surface of X to be directed inward, as shown in Fig. 12. The magnitude of the E.M.F. would increase with the concentration, hence the outermost arrow should be longer at C than at A and the current should flow through the electrometer from A to C (as shown by the horizontal arrow in Fig. 12). This is in accord with the experimental results which show that the dilute solution is positive not only with KCl but with many other salts. To judge from the literature this is the usual condition[34] (but it does not seem to be the case with *Valonia*).

It follows that if we lower the concentration of the solution at A we shorten the length of the arrow at a and if we could make it equal to that at b we should have $a - b = 0$ and we could then learn the value of $c - d$. Whether we can tell when such a state is reached must be decided by future investigation.

The study of the concentration effect may be of value as throwing light on the nature of protoplasm. According to some authors a salt may give no concentration effect with certain substances immiscible with water[28] but with others[35,36] the dilute solution appears positive and with still others negative. If this be the case we may hope by such studies to gain some idea of the nature of the substance constituting the protoplasmic surface.

SUMMARY.

A method distinguishing between the concentration effect due to the cell wall and that due to the protoplasm is described: the importance

[34] *Cf.* Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 6th edition, 1926.

[35] *Cf. e.g.* Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920.

[36] *Cf.* Cremer, M., in Bethe, A., *et al.*, Handbuch der normalen und pathologischen Physiologie, Berlin 1928, viii, 999.

of this lies in the fact that if the protoplasm shows a concentration effect one or both ions of the salt must tend to enter its outer surface.

Studies on the concentration effect of KCl with living protoplasm of *Nitella* show that when P.D. is plotted as ordinates and the logarithm of concentration as abscissæ the graph is not the straight line demanded in the ideal case by theory but has less slope and is somewhat concave to the axis of the abscissæ.

With a variety of salts the dilute solution is positive, which indicates that the cation has a greater mobility in the protoplasm than the anion or that the partition coefficient of the cation (A_c) increases faster than that of the anion (A_a) as the concentration increases. If the result depended on the partition coefficients we should say that when $A_c \div A_a$ increases with concentration the dilute solution is positive. When $A_c \div A_a$ decreases as the concentration increases the dilute solution is negative. In either case the increase in concentration may be accompanied by an increase or by a decrease in the relative amount of salt taken up. Theoretically therefore there need be no relation between the sign of the dilute solution and the relative amount of salt taken up with increasing concentration.

Hypothetical diagrams of the electrical conditions in the cell are given.

If we define the chemical effect as the P.D. observed in leading off at two points with equivalent concentrations of different salts we may say that the chemical effect of the protoplasm is very much greater than that of the cell wall.

CHEMICAL ANTAGONISM OF IONS.

IV. Effect of Salt Mixtures on Glycine Activity.

By HENRY S. SIMMS.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Accepted for publication, April 15, 1929.)

I.

INTRODUCTION.

The three previous papers of this series[1] demonstrated that Na^+ ions counteract the anomalous effect of Mg^{++} ions on the activity of oxalate di-ions ($C_2O_4^-$); and also that Cl^- ions counteract the effect of SO_4^- ions on oxalate di-ions. Furthermore, NaCl, KCl, or $MgCl_2$ each affect gelatin activity in the same manner when only one of these salts is present. Each causes a *lowering* of the pH. However, the addition of a small amount of NaCl or KCl to a gelatin solution already containing another of the above salts causes a very marked *rise* in pH (an effect opposite to that produced by one salt alone). This occurs up to 0.10 molar Na^+ or K^+ ion concentration. Further additions cause a sharp lowering of pH up to 0.15 molar Na^+ or K^+. Beyond that there is little effect.

The analogy of these observations to physiological antagonisms is obvious. In order to investigate the mechanism it is desirable to see if still simpler substances than gelatin behave in the same way. In the present paper it will be shown that NaCl, KCl, $MgCl_2$, and $CaCl_2$, singly and in mixtures, affect the activity of glycine (a simple amino acid) in practically the same manner that they affect gelatin activity.

II.

RESULTS.

The results obtained in these experiments are obvious from the accompanying figures. Fig. 1 shows the influence of salts on the pH

[1] Simms, H. S., *J. Gen. Physiol.*, 1928, xii, 241, 259; 1929, xii, 511.

of a 0.0100 molar solution of glycine containing one-half equivalent of NaOH. NaCl alone or KCl alone *lower* the pH in the manner shown. However, if we start with 0.00416 molar KCl and add increasing amounts of NaCl, we get first a *rise* in pH until 0.007 M NaCl is present. Then there is a sharp break and further additions *lower* the pH until 0.035 M NaCl is present. Further additions of NaCl *increase* the pH as shown.

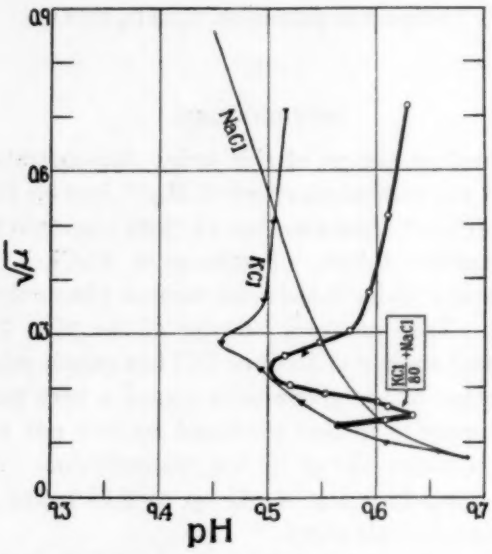

FIG. 1. Antagonistic effects of KCl + NaCl mixtures compared with the effects of KCl or NaCl alone on the pH of glycine solutions (containing 0.5 equivalent of NaOH). Note that both cations are monovalent.

Fig. 2 shows that the same phenomena take place when NaCl is added to glycine solutions containing $MgCl_2$. The breaks come at 0.015 molar and 0.085 molar NaCl concentration *regardless of the amount of $MgCl_2$ present.* This is the same result that was observed with gelatin.

Fig. 3 shows that $CaCl_2$ + NaCl mixtures behave essentially the same as $MgCl_2$ + NaCl mixtures and KCl + NaCl mixtures.

Fig. 4 shows the effect of adding $CaCl_2$ to glycine solution containing $MgCl_2$. The same sort of curve is produced as is found with the other

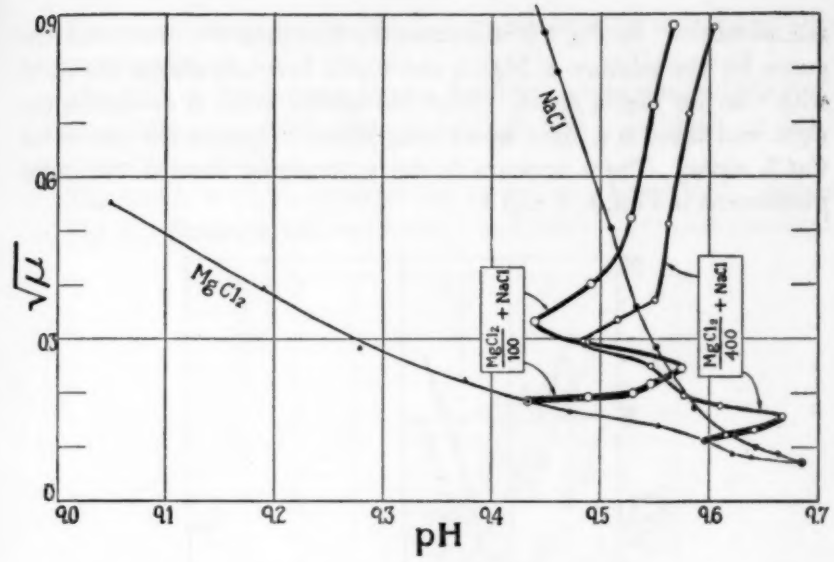

FIG. 2. Effect of $MgCl_2$ + NaCl mixtures on the pH of glycine solutions, compared with the effect of each salt alone. One divalent and one monovalent cation.

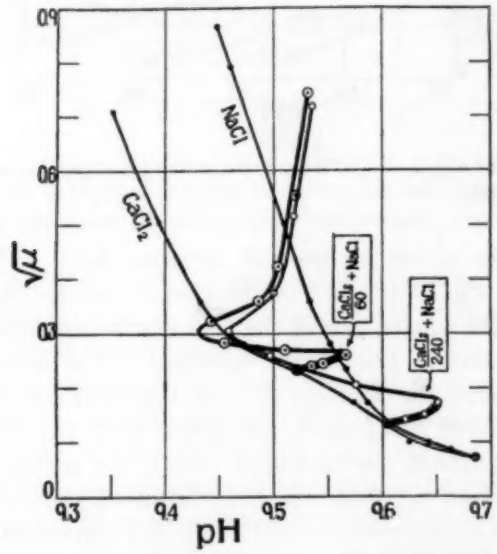

FIG. 3. Effect of $CaCl_2$ + NaCl mixtures on the pH of glycine solutions, compared with the effect of each salt alone. One divalent and one monovalent cation.

salt mixtures. In Fig. 4 it is noteworthy that after the first break the curve for the mixture of $MgCl_2$ and $CaCl_2$ becomes almost identical with that for $MgCl_2$ alone. After the second break it swings to the right and there is a third break near where it crosses the curve for $CaCl_2$ alone. There appears to be a tendency toward the same phenomena in Figs. 1, 2, and 3.

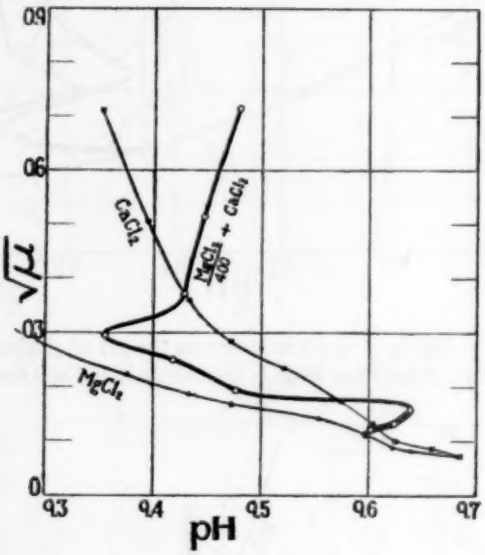

FIG. 4. Effect of $MgCl_2$ + $CaCl_2$ mixtures on the pH of glycine solutions. Both cations are divalent.

In Fig. 5 the curves for the salt mixtures KCl + NaCl; $MgCl_2$ + NaCl; and $CaCl_2$ + NaCl are plotted in another manner. The ordinates are the square root of the Na^+ ion concentration (including the NaOH added to the mother solution). The abscissas are the difference between the observed pH and that obtained with the same ionic strength with NaCl alone. In other words the curve for NaCl is taken as a standard, the deviations from which are plotted in Fig. 5.

It will be observed that the first break of each curve (except the KCl + NaCl curve) comes at 0.015 M NaCl concentration *regardless of the amount of the other salt.*

The second break for the $MgCl_2$ curves is at 0.085 M NaCl and is a little lower for the $CaCl_2$ curves.

The KCl + NaCl curve has its breaks at about one-half the NaCl concentrations at which they occur with $MgCl_2$ + NaCl, or $CaCl_2$ + NaCl.

The $MgCl_2$ + $CaCl_2$ curve (of Fig. 4) was not plotted in the manner of Fig. 5. However, the first break is at 0.005 M Ca^{++} and the second

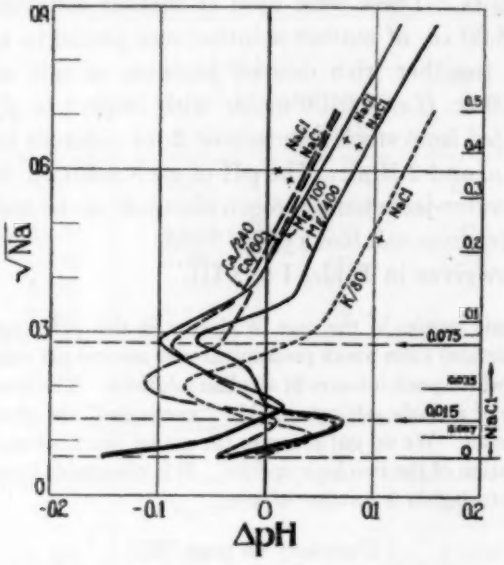

FIG. 5. Effect of NaCl in the presence of another salt, relative to the effect of NaCl alone. The breaks are independent of the amounts of the other salts.

break is at 0.025 M Ca^{++}. It was stated above that the breaks with $MgCl_2$ + $NaCl_2$ were at 0.015 M and 0.085 M NaCl, respectively. These two sets of values do not agree.

If, however, we convert the ion concentrations into "ionic strength" values we have the breaks with $MgCl_2$ + NaCl coming at 0.015 μNaCl and 0.085 μNaCl, while with $MgCl_2$ + $CaCl_2$ they come at 0.015 μCaCl and 0.075 $\mu CaCl_2$. Apparently the position of the breaks is a function of the ionic strength of the added salt rather than of its concentration.

These results with glycine cannot be explained on the basis of colloidal phenomena. They tend to support the mechanism suggested

in the previous paper of the series, namely, that cations in mixtures selectively inactivate the two ionic species[2] to degrees not proportional to the inactivation by one cation alone.

III.

EXPERIMENTAL.

0.02 molar glycine plus 0.500 equivalent of NaOH was made up in 100 cc. amounts. These were used as mother solutions. For each observation 5.00 cc. of mother solution was placed in a 10 cc. volumetric flask, together with desired amounts of salt solutions, and made up to 10 cc. (*i.e.*, 0.0100 molar with respect to glycine). The salts were added from stock solutions of three different ionic strengths ($0.025\,\mu$, $0.25\,\mu$, and $2.50\,\mu$). The pH of each solution was measured at 25°C. in a water-jacketed hydrogen electrode of the bubbling type.

Solutions free from salt had a pH of 9.685.

The data are given in Tables I to VIII.

[2] The two ionic species in the case of glycine in this pH range are, first the *neutral* (or zwitterion) form which predominates at neutral pH values, and second the *anion* form which predominates in alkaline solutions. The lowering of the pH on the addition of a single salt presumably "inactivates" the glycine anion more than the zwitterion. We do not measure the actual inactivation, but rather the ratio of inactivation of the two ionic species. It is reasonable to suppose that this ratio may be disturbed in a mixture of salts.

(Summary on page 792.)

TABLE I.

Effect of NaCl on Na Glycinate.

($\mu = C_{NaCl} + 0.005$.)

C_{NaCl}	pH	C_{NaCl}	pH	C_{NaCl}	pH
0	9.685	0.050	9.567	0.625	9.460
0.0025	9.663	0.075	9.551	0.750	9.448
0.0050	9.643	0.125	9.533	1.000	9.416
0.0250	9.587	0.250	9.510		

TABLE II.

Effect of KCl on Na Glycinate.

($\mu = C_{KCl} + 0.005$.)

C_{KCl}	pH	C_{KCl}	pH	C_{KCl}	pH
0	9.685	0.025	9.526	0.250	9.504
0.0025	9.558	0.050	9.494	0.500	9.516
0.0050	9.609	0.075	9.457		
0.0125	9.565	0.125	9.497		

TABLE III.

Effect of $MgCl_2$ on Na Glycinate.

($\mu = 3\ C_{MgCl_2} + 0.005$.)

C_{MgCl_2}	pH	C_{MgCl_2}	pH	C_{MgCl_2}	pH
0	9.685	0.0050	9.553	0.0250	9.279
0.00050	9.638	0.0075	9.472	0.0500	9.191
0.00100	9.621	0.0100	9.433	0.100	9.049
0.00250	9.596	0.0150	9.376		

TABLE IV.

Effect of $CaCl_2$ on Na Glycinate.

($\mu = 3\ C_{CaCl_2} + 0.005$.)

C_{CaCl_2}	pH	C_{CaCl_2}	pH	C_{CaCl_2}	pH
0	9.685	0.0083	9.574	0.083	9.394
0.00083	9.658	0.0167	9.521	0.167	9.352
0.00167	9.624	0.0250	9.472		
0.00417	9.604	0.0417	9.433		

TABLE V.

Effect of NaCl on a Glycine Solution Containing 0.0125 M KCl.

(pH without salt = 9.685.)

($\mu = C_{NaCl} + 0.0175$.)

C_{NaCl}	pH	ΔpH	C_{NaCl}	pH	ΔpH
0	9.565	−0.045	0.050	9.516	−0.042
0.0025	9.609	+0.005	0.075	9.577	+0.031
0.0050	9.634	+0.034	0.125	9.594	0.064
0.0125	9.601	+0.014	0.250	9.611	0.104
0.0250	9.521	−0.056	0.500	9.628	0.155

TABLE VI.

Effect of NaCl on Glycine Solutions Containing $MgCl_2$.

(pH without salt = 9.685.)

C_{NaCl}	pH	ΔpH	C_{NaCl}	pH	ΔpH
A. 0.00250 M $MgCl_2$ plus various amounts of NaCl ($\mu = C_{NaCl} + 0.0125$).					
0	9.596	−0.034	0.075	9.484	−0.064
0.0025	9.616	−0.003	0.100	9.516	+0.021
0.0050	9.641	+0.029	0.125	9.550	+0.020
0.0125	9.667	+0.070	0.250	9.563	+0.056
0.0187	9.610	+0.023	0.500	9.582	+0.110
0.0250	9.577	−0.003	0.750	9.604	+0.157
0.0500	9.546	−0.016			
B. 0.0100 M $MgCl_2$ plus various amounts of NaCl ($\mu = C_{NaCl} + 0.035$).					
0	9.433	−0.151	0.075	9.440	−0.099
0.0025	9.489	−0.093	0.125	9.491	−0.034
0.0050	9.530	−0.048	0.250	9.528	+0.022
0.0125	9.547	−0.025	0.500	9.548	+0.078
0.0250	9.575	+0.012	0.750	9.567	+0.122
0.0500	9.486	−0.062			

TABLE VII.

Effect of NaCl on Glycine Solutions Containing $CaCl_2$.

(pH without salt = 9.685.)

C_{NaCl}	pH	ΔpH	C_{NaCl}	pH	ΔpH
A. 0.00416 M $CaCl_2$ plus various amounts of NaCl ($\mu = C_{NaCl} + 0.0175$).					
0	9.604	−0.006	0.050	9.497	−0.061
0.0025	9.621	+0.017	0.075	9.458	−0.088
0.0050	9.638	+0.038	0.125	9.500	−0.030
0.0125	9.651	+0.064	0.250	9.518	+0.011
0.0250	9.575	−0.002	0.500	9.536	+0.063
B. 0.0167 M $CaCl_2$ plus various amounts of NaCl ($\mu = C_{NaCl} + 0.050$).					
0	9.521	−0.046	0.050	9.443	−0.097
0.0025	9.535	−0.030	0.075	9.486	−0.047
0.0050	9.546	−0.017	0.125	9.504	−0.018
0.0125	9.567	+0.010	0.250	9.520	+0.019
0.0187	9.511	−0.021	0.500	9.532	+0.064
0.0250	9.455	−0.096			

TABLE VIII.

Effect of $CaCl_2$ on Na Glycinate Solutions Containing 0.0025 M $MgCl_2$.

(pH without salt = 9.685.)

($\mu = 3\ C_{CaCl_2} + 0.0125$.)

C_{CaCl_2}	pH	C_{CaCl_2}	pH	C_{CaCl_2}	pH
0	9.596	0.0083	9.476	0.083	9.447
0.00083	9.602	0.0167	9.418	0.167	9.479
0.00167	9.624	0.025	9.355		
0.00417	9.639	0.0417	9.428		

SUMMARY.

The pH of a 0.01 molar solution of glycine, half neutralized with NaOH, is 9.685. Addition of only one of the salts NaCl, KCl, $MgCl_2$, or $CaCl_2$ will *lower* the pH of the solution (at least up to 1 μ).

If a given amount of KCl is added to a glycine solution, the subsequent addition of increasing amounts of NaCl will first *raise* the pH (up to 0.007 M NaCl). Further addition of NaCl (up to 0.035 M NaCl) will *lower* the pH, and further additions slightly raise the pH.

The same type of curve is obtained by adding NaCl to glycine solution containing $MgCl_2$ or $CaCl_2$ except that the first and second breaks occur at 0.015 M and 0.085 M NaCl, respectively.

Addition of $CaCl_2$ to a glycine solution containing $MgCl_2$ gives the same phenomena with breaks at 0.005 M and 0.025 M CaCl; or at *ionic strengths* of 0.015 $\mu CaCl_2$ and 0.075 $\mu CaCl_2$. This indicates that the effect is a function of the ionic strength of the added salt.

These effects are sharp and unmistakable. They are almost identical with the effects produced by the same salt mixtures on the pH of gelatin solutions. They are very suggestive of physiological antagonisms, and at the same time cannot be attributed to colloidal phenomena.

THE EFFECT OF SUDDEN CHANGES OF TEMPERATURE ON PROTOPLASMIC STREAMING.

By S. F. COOK.

(From the Division of Physiology, University of California, Berkeley.)

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I.

In the numerous studies which have been made concerning the effect of temperature on various cellular activities many interesting irregularities in the conduct of cells and tissues have been brought to light. An example of such phenomena is the sudden cessation of protoplasmic streaming in *Nitella* which occurs when the temperature is suddenly lowered. This response to temperature change has been discussed recently by Lambers (1, 2), who finds that in case of a sudden transition from hot to cold there is a "shock" which causes the streaming to stop instantly. There is then a recovery but the rate of movement normal for a given temperature is not attained for 10 to 20 minutes.

A similar inhibition is known to take place if the *Nitella* cell is stimulated mechanically, and Fitting (3) has shown that a sudden increase of light intensity brings about the same effect. There are therefore at least three distinct types of stimuli which cause an instantaneous inhibition of streaming in *Nitella*. A brief investigation of the inhibition due to change in temperature has been made and the results are here reported.

II.

The material used was *Nitella clavata* which had been secured from a pond in Golden Gate Park, San Francisco, and had been kept in tanks. Single cells were cut out, placed in petri dishes and examined under the low power of the microscope. The rate of streaming was measured by means of a micrometer eye-piece and a stop watch. Individual determinations were made of the time necessary for a particle to travel between two selected points in the eye-piece scale.

Since the same objective and eye-piece were always used, the measurements on a large number of cells are comparable. The light source was a 40 watt Mazda bulb placed at a fixed distance from the microscope. This insured a constant illumination and a constant light intensity through the condenser. For lower temperatures the cells were immersed in mixtures of ice and water, the proportions of which were so regulated that by frequent stirring a constant temperature might be obtained. The higher temperatures were maintained by keeping the room at approximately the desired point and then using mixtures of warm and cool water. The error of this method was no greater than 0.1°, and since no intervals of less than 5° were investigated (usually the intervals were at least 10°) this error is without significance. In fact the individual variability of the cells is much greater than any error of the method.

The results may be expressed as microns per second. But since most of the data are of a comparative nature there is no need of introducing absolute units. All rates may be expressed as per cent of the normal rate, the latter being taken arbitrarily as the rate at any given temperature.[1]

It may be noted in passing that the sensitivity of *Nitella* to mechanical disturbance, mentioned above, is very great. A small jar or blow, such as striking against any hard object, or a bending of the cell, will stop the streaming completely. Special care must therefore be exercised in handling the cells, particularly in transferring them from one dish to another. In these experiments the following precaution was always taken. Before a cell was immersed in water at a new temperature, it was transferred to a second container with water at the previous temperature. If this operation had no effect on the streaming, the cell was then placed at the new temperature. Furthermore a special series of control experiments showed that with due care cells could be moved back and forth from one petri dish to another indefinitely without affecting the rate of streaming.

[1] The extraordinary constancy of the rate of streaming in *Nitella*, at constant temperature, is worthy of note. Measurement of 73 cells showed an average normal rate at 20° of 73.1 μ per second. The maximum deviation was 21.3 per cent and the average deviation was 5 per cent, or 3.65 μ per second.

III.

If *Nitella* cells are carried from 20°C. to 35°C., there is no cessation of the streaming. There is of course a change of rate, from that characteristic of 20° to that of 35°. This change is very nearly instantaneous. Usually 20 to 30 seconds elapse before the first record can be made, representing time unavoidably lost in manipulating the cell, getting it under the microscope, and focussing. During this half minute or less the new rate has become established. Probably the actual time consumed in bringing the streaming to the new speed is a matter of a few seconds. A similar situation obtains if a cell is allowed to remain at 1° for 30 minutes or more and is then brought to 20°.

The result may be very different when the temperature is lowered abruptly. If the drop is from 35° to 20° the flow continues without interruption but at a slower rate, *viz.*, that rate characteristic of 20°. The effect of such a fall in temperature is similar to that of a rise, since it causes an immediate change in rate; immediate in the sense that the rate becomes constant within 20 seconds. In fact a cell may be alternated between 35° and 20° indefinitely with no change except in the rate of streaming. Suppose, however, that we place the cell in water at 10° instead of 20°. In this case the streaming stops completely and does not begin again for at least 2 minutes. This instantaneous and complete cessation is a very different phenomenon from the mere change of speed observed between 35° and 20°. The difference is also exemplified in the following experiment. If a cell is taken from water at 35°, rapidly dipped into water at 10° and replaced at 35° the streaming likewise is seen to have completely stopped. The exposure to the temperature of 10° may not last longer than half a second. Yet the effect is observed. The stimulus, then, is clearly the sudden transition from the high to the low temperature and not a prolonged exposure at the low temperature.

Another series of experiments extends the above results and demonstrates that the fall in temperature must exceed a certain definite number of degrees in order to produce the effect. In this case the initial temperature was 20°. The cells were dipped for approximately half a second in water at several lower temperatures and were returned to 20° for observation. The fall from 20° to 15° does not stop the

streaming, nor does that from 20° to 10°. But passing from 20° to 1° the streaming ceased in all cells observed. The transition, or critical point, for an initial temperature of 20° then lies between 1° and 10°. In an attempt to locate this critical point more exactly, two facts became evident.

1. There is a decided variability among the cells with respect to the temperature at which they stop streaming. This is shown in Table I and Fig. 1. With an initial temperature of 20° a number of cells were exposed for less than a second to temperatures varying from 3° to 7°. It was found that in all cells the streaming stopped at 3°, but at the other temperatures (4°, 5°, 7°) only part of the cells were so affected. Plotting per cent not affected against temperature we obtain Fig. 1. From these data it may be concluded that for a large number of *Nitella* cells there is a general range of temperature which causes a cessation of streaming.

TABLE I.

Effect of Bringing Cells from 20° to Various Temperatures for 0.5 Second.

Temp.	No. cells	No. cells unaffected	Per cent of cells unaffected
10°	20	20	100
7°	44	36	82
5°	59	40	77
4°	40	14	35
3°	20	0	0

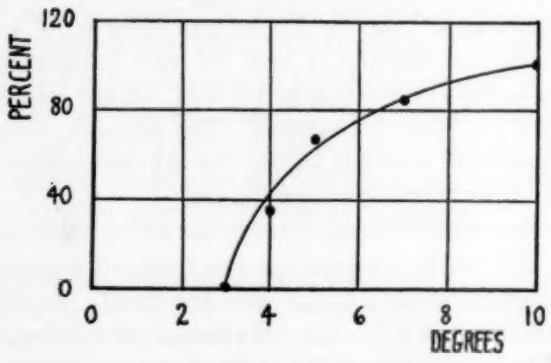

FIG. 1. This figure shows the relative number of cells in which the streaming is inhibited when the initial temperature is 20°C. The ordinates are per cent of the total number in which there is no inhibition. The abscissae indicate the low temperature to which the cells were exposed.

2. Nevertheless there exists for every single cell some quite definite critical temperature. In all the cases examined there was no single instance where a partial cessation occurred. The streaming either continued in a normal manner or was inhibited completely.

The data presented thus far permit one more conclusion to be drawn. The inhibition of streaming does not occur at one particular point on the temperature scale regardless of the original temperature, but is rather a function of the temperature gradient. It may be pointed out again that, regardless of the variability of individual cells, a drop from 35° to 10° causes 100 per cent inhibition whereas a drop from 20° to 10° causes no inhibition whatever. Were there any physical or chemical state inherent in the condition of the cell adjusted to 10° which caused an inhibition of streaming, then that state would have to exist regardless of the previous temperature. But since the effect of exposure to 10° is 100 per cent different depending on the previous temperature we are forced to the conclusion that it is the sudden fall of temperature through at least a minimum number of degrees which gives rise to the observed effects. The temperature range with which it is possible to work is too limited to permit an exact quantitative determination of this minimum distance for all initial temperatures, but it may be stated that probably a fall of 15° to 20° is sufficient to stop streaming.

Certain theoretical considerations are pertinent at this juncture. The immediate increase in the rate of streaming with sudden rise in temperature, and, within the prescribed limits, the corresponding decrease with fall of temperature, point to the acceleration and retardation of a chemical reaction system. Since the streaming in these cells is considered to be governed by their metabolism it is entirely reasonable that these particular effects of temperature should be obtained. It is what might be expected of a chemical system. But the discontinuity introduced by the sudden cessation of streaming below the critical temperature cannot be explained on such a basis. It is very unlikely that a chemical reaction can be brought to a complete stop by a fall of 20°. This view is substantiated by the fact that there is no intergradation between moderate retardation and complete inhibition. We must therefore fall back on the supposition that the inhibition is a phenomenon which has no connection with the metabolism of the cell

and which is connected with the physical properties of the protoplasmic system.

IV.

If cells are transferred from 20° to 1° and allowed to remain at 1° for varying periods before being returned to 20° there is a distinct influence of the exposure time on the recovery of the normal rate. This is illustrated in Figs. 2 to 5. Each figure represents data from several cells. The individual variability causes considerable dispersion of the points, but if enough cells are studied it is found that the dispersion falls within definite limits, forming a band of points instead of a single curve. For practical purposes, however, a curve may be drawn along the midline of the band which will represent an average of all the points.

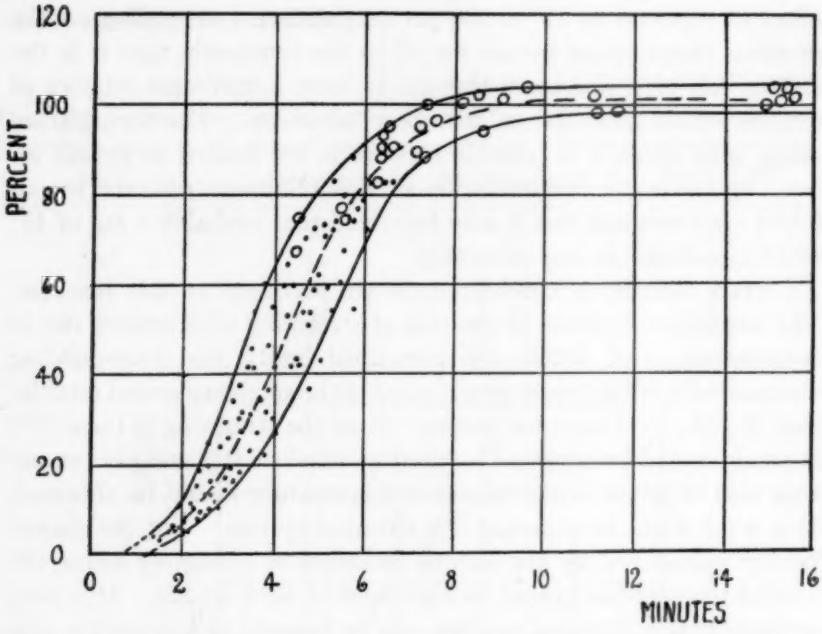

FIG. 2. The course of recovery of streaming in 9 cells after 0.5 second exposure to 1°C. The initial temperature was 20°C. and the cells were replaced at this temperature for measurement. The ordinates denote per cent of the normal rate, the abscissae time in minutes. The dots represent single measurements, the circles represent averages of at least three measurements.

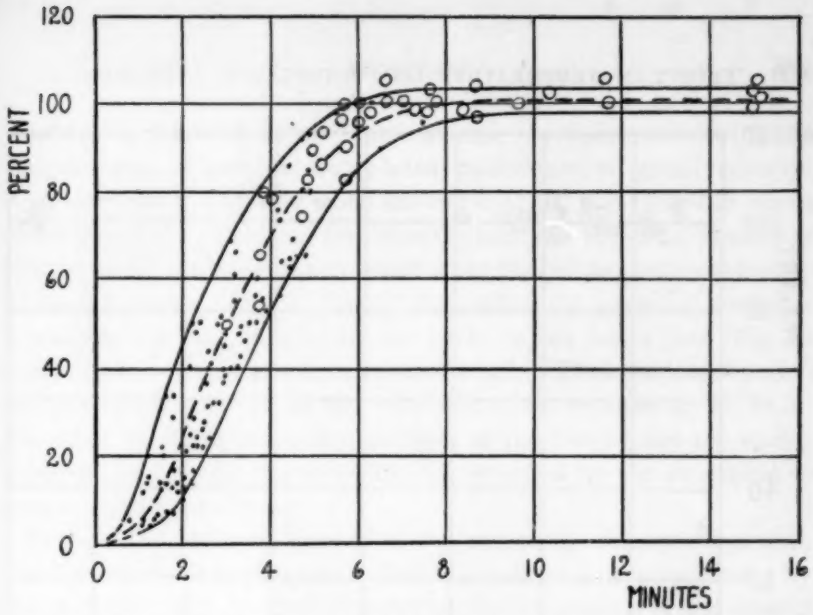

FIG. 3. The course of recovery of 10 cells after 5 minutes exposure to 1°C. Initial temperature 20°C. Ordinates and abscissae as in Fig. 2.

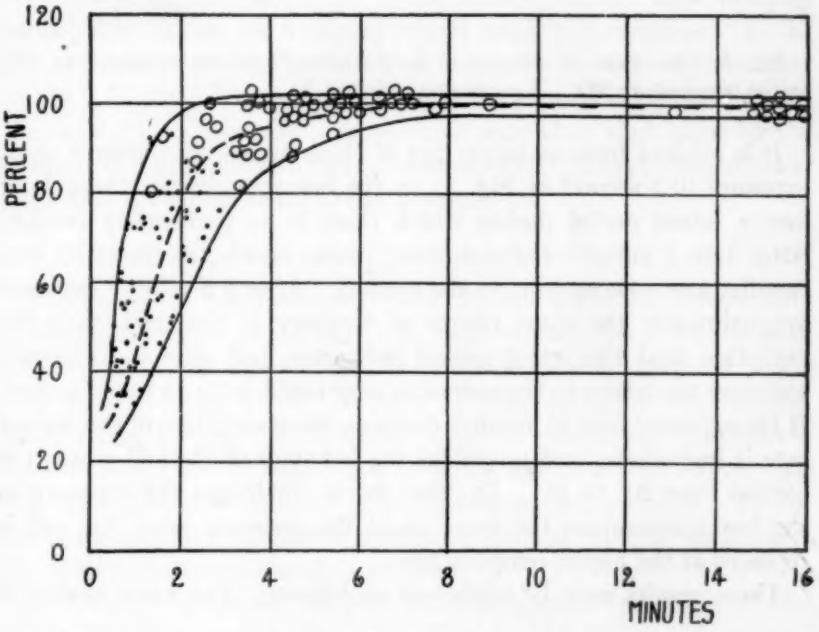

FIG. 4. The course of recovery of 12 cells after 10 minutes exposure to 1°C. Initial temperature 20°C. Coordinates the same as Fig. 2.

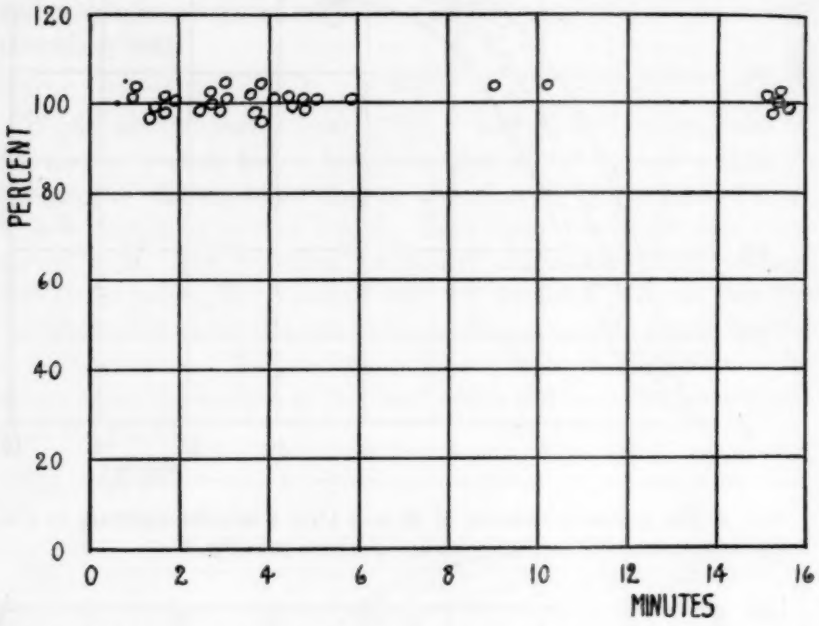

FIG. 5. The course of recovery of 6 cells after 20 minutes exposure to 1°C. Initial temperature 20°C. Coordinates as in Fig. 2.

It is evident from an inspection of these figures that after a short exposure (0.5 second in Fig. 2) to the low temperature there is at first a latent period during which there is no perceptible motion. After 1 to 2 minutes the streaming begins slowly, increases its rate rapidly, and soon returns to the normal. After a 5 minute exposure approximately the same course of recovery is observed, with the exception that the latent period is shorter, but after a 10 minute exposure the return to the normal is very rapid, with no latent period. If the exposure is of 20 minutes duration the resumption of the normal rate is immediate, and resembles the behavior of the cell when it is carried from 20° to 35°. In other words the longer the exposure to the low temperature the more rapid the recovery when the cell is replaced at the higher temperature.

These results may be explained as follows. The same course of

recovery is followed at all temperatures; the rate varies with the temperature. There is at first a latent period prior to actual recovery. This lasts about a minute when the cell is at 20° and probably about 5 minutes at 1°. Evidence for this is the fact that the initial absence of motion at 20° continues much longer when the cell has been exposed to 1° for 0.5 second (Fig. 2) than it does when the exposure period is 5 minutes. In fact, judging by the curve in the latter case (Fig. 3) there can be scarcely any latent period at all. Thus the latent period has practically come to an end when the cell is replaced at 20°. On the other hand the remaining portions of the two curves are nearly identical, and nearly the same time is consumed for the streaming to return to the normal rate.

If the cell is removed from 1° at the end of 10 minutes the actual, rapid, recovery is in progress. This is demonstrated quite clearly by the fact that (Fig. 5) after 20 minutes the resumption of the normal rate for 20° is immediate. Therefore we may say that after the streaming has been brought to a complete standstill by the sudden exposure to a temperature of 1° the recovery starts after a latent period, and follows an S-shaped course until it is complete. If the cell remains at 1° this sequence of events is carried out relatively slowly. If the cell at any time during the process is replaced at 20° the recovery proceeds at a more rapid rate than that prior to the moment of replacement. Hence recovery at 20° is *apparently* quicker the longer the exposure to the low temperature.

V.

The statement was made previously that the inhibition of streaming at low temperatures might have a physical explanation. The data on recovery afford two more lines of evidence concerning this point.

1. Even though, at 1°, recovery is proceeding, a very careful examination for over 30 minutes failed to detect any perceptible motion in the protoplasm. Recovery of the *power to stream* can thus take place while there is no *actual streaming* in progress. Therefore the two factors, the actual streaming, and the capacity for streaming, must be entirely separate and distinct.

2. We may determine roughly the temperature coefficient of the

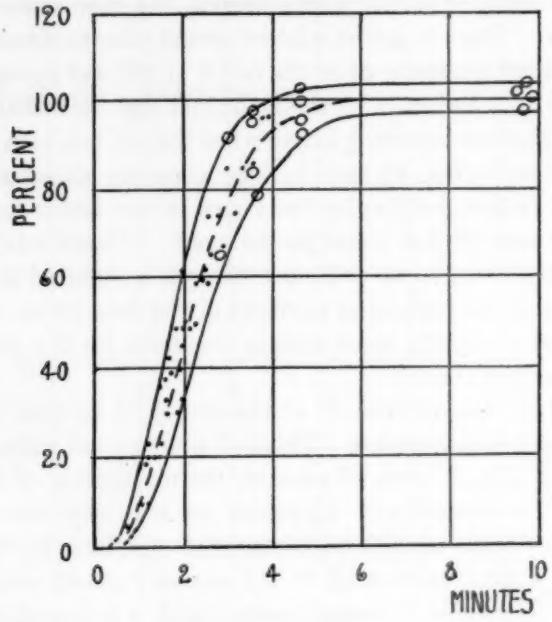

FIG. 6. The course of recovery of 5 cells after 0.5 second exposure to 1°C. The initial temperature was 35°C. and the measurements were made at 35°C. Coordinates as in Fig. 2.

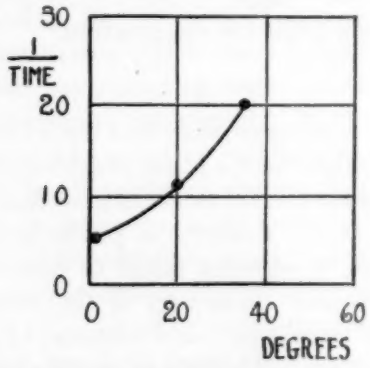

FIG. 7. The relation between time for complete recovery and the temperature maintained during recovery made. In all cases there was an exposure of 0.5 seconds to 1°C. The ordinates represent the time necessary to recover and the abscissae the temperature. See text.

recovery process, using a Q_{10} value. Fig. 6 shows the recovery from 0.5 second exposure to 1° when the preceding temperature was 35°. The recovery is much more rapid than at 20° and its duration may be taken at approximately 5 minutes. From Fig. 2 the duration of recovery with 20° is about 8 minutes. That with 1° may be considered 20 minutes, because it is at the end of this time that immediate resumption of streaming in 20° takes place. If we take the reciprocals of these times as representing the relative velocities and plot them against temperature we get Fig. 7. Using the Van't Hoff formula

$$Q_{10} = \left(\frac{K_2}{K_1}\right)^{\frac{10}{T_1 - T_1}}$$

we find a Q_{10} value of 1.51 between 1° and 20° and 1.45 between 20° and 35°. This coefficient is of the order usually expected of physical phenomena, and to this extent corroborates the idea already advanced that the inhibition and recovery of streaming, as occasioned by a sudden fall of temperature, have a physical basis inherent in the structure of protoplasm.

SUMMARY.

A sudden fall of temperature of 15° to 20° causes a complete cessation of the protoplasmic streaming in *Nitella*. The recovery of the normal rate follows a definite course and the time of recovery is dependent on the temperature. Evidence is adduced to show that the inhibition of streaming is due to a physical phenomenon.

The author wishes to extend his thanks to Professor A. R. Davis and Professor D. R. Hoagland for their kindness in supplying the *Nitella* used in these experiments.

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A GLASS ELECTRODE APPARATUS FOR MEASURING THE pH VALUES OF VERY SMALL VOLUMES OF SOLUTION.

By D. A. MacINNES and MALCOLM DOLE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, May 11, 1929.)

In a recent article[1] the authors have described a new type of glass electrode, which is of convenient size, and at the same time of sufficiently low resistance for accurate work. Such an electrode is shown diagrammatically on the right-hand side of Fig. 1. The lower end of glass tube *A* supports the glass diaphragm *D* which in our experiments has been about 0.001 mm. thick. The tube *A* is partly filled with 0.1 N HCl into which is inserted the silver-silver chloride electrode *B*. More details concerning these electrodes are given in the article referred to above.[2]

Since glass electrodes of this type with glass diaphragms 4 mm. or less in diameter can be used, they seemed admirably adapted to measurements involving very small amounts of material. The need for such measurements arises frequently, especially in biological investigations. The apparatus shown diagrammatically in Fig. 1 was therefore designed to adapt the electrodes to this purpose. The vessel *C* holds a reference saturated calomel electrode. This electrode is connected, through a stopcock, with the reservoir *R* which contains

[1] MacInnes, D. A., and Dole, M., *J. Ind. Eng. Chem.* (Analytical Edition), 1929, i, 57. This paper contains references to the previous work with glass electrodes, with the exception of a recent article by Mirsky and Anson (Mirsky, A. E., and Anson, M. L., *J. Biol. Chem.*, 1929, lxxxi, 581.

[2] Through the kindness of Dr. Alexis Carrel we have tested the utility of these electrodes in connection with tissue culture work. The usual vessel for that work was provided with three outlet arms. One arm contained a tube through which the medium could be drawn to make contact with a saturated calomel electrode, and the other two arms held glass electrodes. One glass electrode was kept in the medium and the other in the immediate vicinity of the tissue studied. In this way it was possible to follow accurately the change of pH with time of the tissue and the medium separately.

a supply of saturated KCl solution. Another branch of the tubing connects with the tip *T*. An additional branch tube *F* is closed by a piece of rubber tubing (a so-called "policeman") on which a screw pinchcock is placed. The whole apparatus is mounted on adjustable screw clamps, as shown in Fig. 2, so that the glass electrode and the calomel electrode with its attachments can be independently raised and lowered. The clamp holding the glass electrode is insulated from the rest of the support with a piece of Bakelite.

To make a pH determination the following steps are necessary. The rubber tube on *F* is compressed by the pinchcock. The stopcock below the reservoir

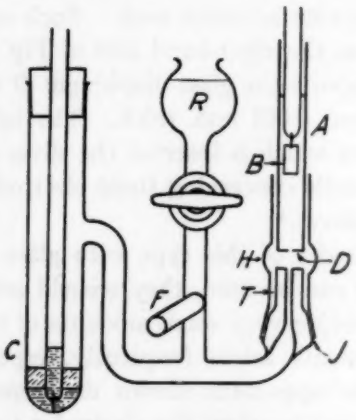

FIG. 1. Diagram of apparatus

R is then opened slightly and saturated KCl is run out on the tip *T*. With a piece of filter paper this solution is removed from the tip so that the solution lies in the capillary and flush with the surface. A drop of the solution whose pH is desired is then placed on the tip. By loosening the pinchcock on *F* slightly this drop is drawn into the capillary tube so that the liquid junction is lowered a few millimeters below the surface of the tip. The remainder of the drop is then removed with filter paper. This procedure has the effect of removing any KCl-bearing solution from the tip. Another drop of the solution under observation is then added, and the protecting tube *H* is put in place as shown. The glass electrode is next lowered, by means of the screw adjustment, until the glass diaphragm *D* comes in contact with the drop. An E.M.F. measurement may then be made with an electrometer and potentiometer.

It is necessary to put a thin coating of paraffin on the tubing up to the edge of

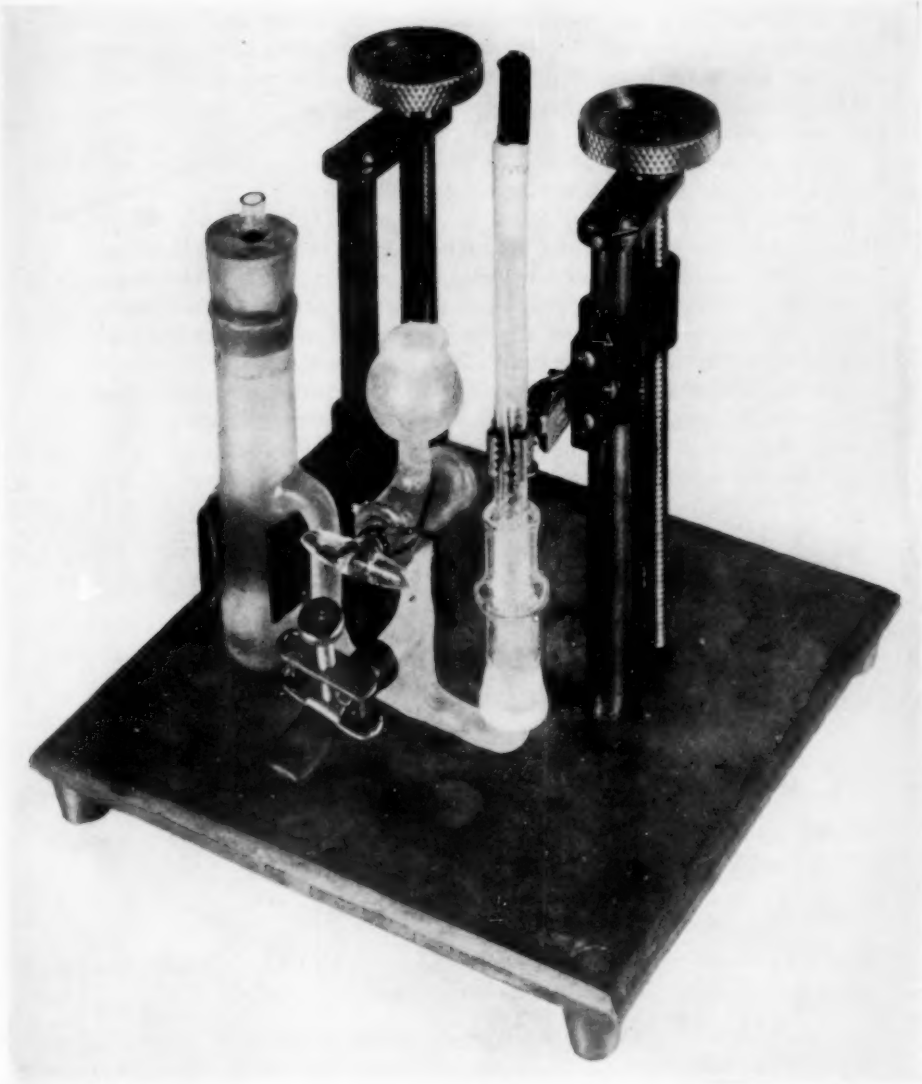

FIG. 2. Glass electrode apparatus for measuring the pH of very small volumes of solution

the tip T to prevent the drop of solution from spreading. A similar coating around the lower edge of the glass electrode is also desirable for the same reason. The protecting tube H was added to the apparatus after finding that the potentials observed were unsteady and drifting due to evaporation from the edge of the drop. It is important to have a close fitting ground joint at J so that rising air currents are prevented.

Our measurements have been made in a constant temperature room, at 25°, with a "Type K" Leeds and Northrup potentiometer, using a Compton electrometer, made by the Cambridge Scientific Instrument Co., as a null instrument. Readings could be made to about 0.2 millivolt. Due to the relatively low resistances of the glass electrodes (about 10 megohms in our most recent measurements) little screening of the electrical system was found necessary. For the same reason ordinary care in insulation was ample. The connections from the potentiometer to the electrometer were made with lead-screened wire.

The formula to be used in computing pH values from the measurements at 25°C. with the apparatus as described is

$$\text{pH} = \frac{E + G + 0.1066}{0.05915}$$

in which E is the measured potential, and G the potential (to be discussed below) which may be present in the glass. The constant 0.1066 can be obtained as follows. The potential of the system

$$\text{Hg} \mid \text{HgCl, sat'd KCl} \parallel 0.1 \text{ N HCl} \mid \text{glass} \mid 0.1 \text{ N HCl, AgCl} \mid \text{Ag} \qquad \text{(A)}$$

will be the same as the combination

$$\underset{\longleftarrow\!\!\!\!\!\!\!\!\!\!\text{B}\!\!\!\!\!\!\!\!\!\!\longrightarrow}{\text{Hg} \mid \text{HgCl, sat'd KCl} \parallel 0.1 \text{ N HCl} \mid \text{H}_2} \; - \; \underset{\longleftarrow\!\!\!\!\!\!\!\!\text{C}\!\!\!\!\!\!\!\!\longrightarrow}{\text{H}_2 \mid 0.1 \text{ N HCl, AgCl} \mid \text{Ag}}$$

if the glass acts as a hydrogen electrode and is the source of no other potential. The potential of the cell B is,[3] at 25°,

$$E = 0.2458 - 0.05915 \log a_H$$

in which a_H is the activity of the hydrogen ion in 0.1 N HCl. The cell C has the potential −0.3524.[4] The total potential is therefore

$$E = 0.2458 - 0.3524 - 0.05915 \log a_H \qquad \text{(D)}$$

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[3] Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 1928, p. 672.

[4] From the work of Scatchard, G., *J. Am. Chem. Soc.*, 1925, xlvii, 641, involving a slight interpolation.

Now if we raise the hydrogen ion activity of the acid solution nearest the calomel electrode in the cell A to unity a potential will arise at the glass surface equal to

$$E = +0.05915 \log a_H \qquad (E)$$

so that the new potential will be equal to the sum of D and E or

$$E_0 = 0.2458 - 0.3524 = -0.1066$$

which is the constant in the equation

$$E = E_0 - 0.05915 \log a_H = E_0 + 0.05915 \text{ pH}$$

or the potential of the cell A when the hydrogen ion activity between the glass and the saturated KCl solution is unity.

The potential G in the glass may be determined by placing the glass electrode (with the solution and silver-silver chloride electrode) in a beaker containing 0.1 N HCl and another silver-silver chloride electrode. In our more recent work we have used glass of a composition which gives membranes in which this potential is nearly zero. This and other information we have obtained concerning the relation of the composition of glass to its behavior when made into electrodes will be published elsewhere.

A number of tests were made to see whether the apparatus as described would yield correct pH values. For this purpose four glass electrodes were used with a buffer solution of pH 7.76, as determined by the hydrogen electrode. The results are shown in Table I.

TABLE I.

Electrode	Date	pH
1	Apr. 24	7.84
2	" 24	7.74
3	" 24	7.77
3	" 24	7.75
3	" 25	7.76
4	" 25	7.76
1	" 25	7.76
1	May 2	7.74

Except for Electrode 1 (which initially gave an error of 0.08 pH unit) the measurements are all within 0.02 unit (about 1.2 millivolts).

Extensive tests (not made with this apparatus) have shown that

electrodes, made with the glass we have found most suitable, begin to deviate 0.02 pH unit from the correct values at pH 9.5 and show rapidly increasing deviations at higher pH values, if the solution measured is 0.1 N in sodium ion. The deviations begin at lower pH values if the sodium ion concentration is greater. In alkaline solutions the potentials may be dependent on the nature of the positive ions present and may vary with time. A more complete description of these tests will appear in another article.

An opportunity for testing the usefulness of this apparatus arose in connection with the work of Dr. Marian Irwin of this Institute. An important question to be investigated was whether the penetration of a basic dye changes the pH value of the vacuolar sap of living cells of *Nitella*. A few drops only of this sap can be conveniently obtained at one time. The question could not be settled by means of the hydrogen electrode since it is "poisoned" by the sap, and by the dye, brilliant cresyl blue, which was used. Such poisonings, which are, in many cases at least, due to irreversible oxidation-reduction potentials, do not appear to have any effect on the glass electrode.[5] Furthermore, the use of hydrogen gas would affect the concentration of CO_2 on which the pH value partly depends. The use of indicators with these dye solutions is obviously impossible. By employing the apparatus described above on samples of the sap prepared by Dr. Irwin it was shown that the entrance of the dye raises its pH value considerably. The results of these experiments will be fully described elsewhere.

[5] So far as we have been able to test the glass electrodes they seem, up at least to pH 9, in dilute salt solutions, to react only to changes in hydrogen ion activity. For instance, the paper referred to ([1]) gives data on the electrometric titration of sulfuric acid in the presence of potassium permanganate. The potentials followed the course to be expected from the change of hydrogen ion activity and were uninfluenced by the strong oxidation potential of the permanganate. On the other hand, the glass electrodes showed no change in potential when ferrous sulfate was titrated with potassium dichromate in the presence of an excess of sulfuric acid. In this case the hydrogen ion concentration remained substantially constant during the titration although there was a change in an oxidation-reduction potential of roughly 0.3 volt. We have other evidence, bearing on this matter, which cannot be conveniently summarized and will be published later.

SUMMARY.

A glass electrode apparatus is described with which pH measurements can be made with as small volumes as 2 drops (about 0.14 cc.) of solution.

Using this apparatus the change of pH of the vacuolar sap of *Nitella*, due to the penetration of brilliant cresyl blue, has been readily followed. The sap and the dye have been found to poison the usual type of hydrogen electrode.

THE VALIDITY OF THE GLASS ELECTRODE IN AMMONIUM CHLORIDE BUFFERS.

By SAMUEL E. HILL.*

(From the Physiological Laboratory, Princeton University, Princeton.)

(Accepted for publication, March 25, 1929.)

In the course of experiments on the cytolysis of luminous bacteria it became necessary to know accurately the pH values of 0.5 M solutions of ammonium salts from about pH 5.0 to pH 9.6. Partly because of the high salt concentration colorimetric methods were insufficiently accurate, especially on the alkaline side. On account of the oxidation of quinhydrone in alkaline solutions the quinhydrone electrode could not be applied to more than one-half of the range of values desired. The statements in the literature as to the action of ammonia as a hydrogen electrode poison are conflicting,[1] and also the hydrogen electrode is too slow to be used conveniently when the number of determinations may exceed 100 in a single experiment. The glass electrode seemed to offer the best solution to the problem, being free of salt errors and of all other errors due to changes in the solution as a result of the examination itself. It is said to be accurate to 0.01 pH.[2] The statements as to the accuracy of the glass electrode in alkaline solutions vary. Brown[3] reports good agreement with the formula $E = 0.058 \log (C_1/C_2)$ from pH 4.0 to pH 10.0. Hughes[4] reports approximate agreement with the hydrogen electrode below pH 11.0 and in another paper[5] states that the electrode values were unaffected by large changes in sodium ion concentration below pH 9.0. Since the writer has occasion to use ammonium solutions above pH 9.0 both in presence and absence of sodium chloride, the

* Fellow in Zoology of National Research Council.

[1] *Cf.* Clark, The determination of hydrogen ions, 3rd ed., 1928, p. 439.

[2] Kerridge, P. M. T., *J. Sci. Ins.*, 1926, iii, 404.

[3] Brown, W. E. L., *J. Sci. Ins.*, 1924, ii, 12.

[4] Hughes, W. S., *J. Am. Chem. Soc.*, 1922, xliv, 2860.

[5] Hughes, W. S., *J. Am. Chem. Soc.*, Feb., 1928, p. 491.

experiments described herein were made to determine the validity of the glass electrode in the class of solutions employed. Since others also may be interested in the use of fairly accurately determined ammonium buffers of relatively high concentration, these experiments are recorded here.

In the dry air of the winter laboratory sulfur was found an excellent insulating material and no trouble was experienced from leakage. The

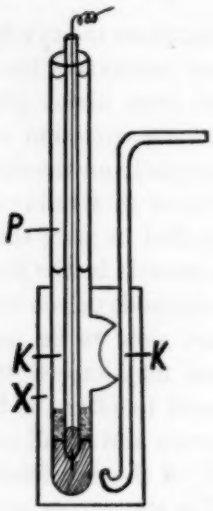

FIG. 1. Glass electrode vessel. K = Saturated KCl. P = Paraffine oil. X = Unknown solution.

form of electrode used is a combination of those of Hughes[4] and Kerridge,[6] a modification suggested by Anson and Mirsky,[7] and further slightly modified by the writer. In the form finally adopted, illustrated in Fig. 1, the delicate glass membrane is protected from the breakage which is inevitable when solutions are pipetted in and out of the cup as suggested by Kerridge,[6] and is to be preferred when 15 or 20 cc. of solution is available for the test. A saturated calomel

[6] Kerridge, P. M. T., *Biochem. J.*, 1925, xix, 611.

[7] Anson, M. L., and Mirsky, A. E., *Proc. Soc. Exp. Biol. and Med.*, 1928, xxv, 346.

electrode is made in the lower part of the glass electrode vessel and the saturated KCl in contact with the glass membrane is standardized daily against M/20 KH phthalate. All calculations are made from its pH of 3.97 by the formula

$$pH_x = 3.97 \pm \frac{E_s - E_x}{0.0581}, \text{ at } 20°,$$

where E_s = p.d. of KH phthalate,
E_x = p.d. of unknown.
A Lindeman electrometer is used as the null instrument.

In the first experiment, 0.5 M ammonium chloride plus 0.5 M ammonia was diluted with successive equal portions of 0.5 M am-

TABLE I.

Activity Coefficients of Several Concentrations of Ammonium Chloride.

f_{a1} is the empirical value, f_{a2} that calculated from Bjerrum's formula.

[NH_4Cl]	[NH_3]	pH	f_{a1}	f_{a2}
0.01	0.00207	8.65	0.8335	0.8532
0.1	0.0207	8.70	0.7437	0.7104
0.25	0.05075	8.73	0.6804	0.6286
0.5	0.1015	8.75	0.6502	0.5564

monium chloride until the concentration of ammonia approached 0.001 M. A similar series was prepared in which 0.5 M ammonium chloride plus 0.5 M sodium chloride plus 0.5 M ammonia was diluted with 0.5 M ammonium chloride plus 0.5 M sodium chloride. The pH values of these solutions were taken and plotted against the logarithm of the concentration of ammonia (Fig. 2). For the ammonia plus ammonium chloride series these values fall on a straight line within the experimental error of the electrode, showing that there is no error due to any change in the electrode at the higher concentrations of hydroxyl ions. For the ammonia plus ammonium chloride plus sodium chloride series the values fall on the same straight line until about pH 8.6, the deviation from this point increasing until there is an error of −0.07 pH at pH 9.4. Considering the experimental error of the method (0.01 pH), corrections are unnecessary below pH 8.6 in the

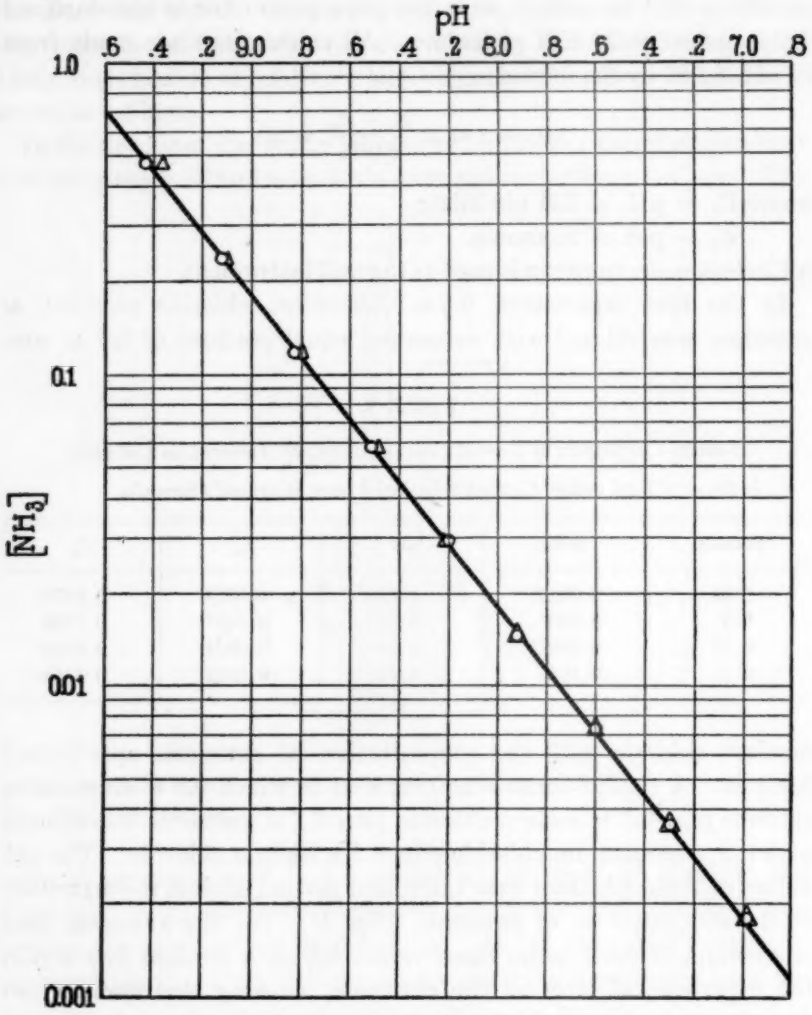

FIG. 2. O = pH of indicated concentration of ammonia in presence of 0.5 M ammonium chloride. Δ = pH of indicated concentration of ammonia in presence of 0.5 M ammonium chloride plus 0.5 M sodium chloride.

series containing sodium chloride and unnecessary at any value as far as determined in the absence of sodium ions (and perhaps the ions of the other alkali metals). For ammonium chloride solutions in presence of 0.5 M sodium chloride the corrections given in Table II are suggested.

In the physiological study in question, the relation of concentration of ammonia to concentration of hydrogen ions is of considerable importance and can be calculated readily if the activity coefficients of the ammonium salts are known. The formulae for calculating these values accurately are of such an involved nature as to be useless for

TABLE II.

Apparent pH of Ammonium Chloride Buffers 0.5 M With Respect to Ammonium Chloride in Presence of 0.5 M Sodium Chloride, With Corrections.

pH	Correction
8.6	+.01
8.8	+.02
9.0	+.03
9.2	+.04
9.4	+.07

the average worker in the biological laboratory.[8] The activity coefficient may be determined empirically from the pH values and the concentrations of ammonia and ammonium chloride. Choosing for an example the sample containing 5.8×10^{-2} mols ammonia per liter, pH 8.51, which happens to give the average value for the series, and applying the buffer equation:

$$[OH^-] = \frac{K_b [NH_3]}{f_a [NH_4]}$$

$$f_a = \frac{1.8 \times 10^{-5} \times 5.8 \times 10^{-2}}{3.24 \times 10^{-6} \times 0.5} = 0.6444$$

The empirical values were determined for several concentrations of ammonium chloride and these are compared in Table I with the

[8] *Cf.* Lewis, G. N., and Randall, M., *J. Am. Chem. Soc.*, 1921, xliii, 1112.

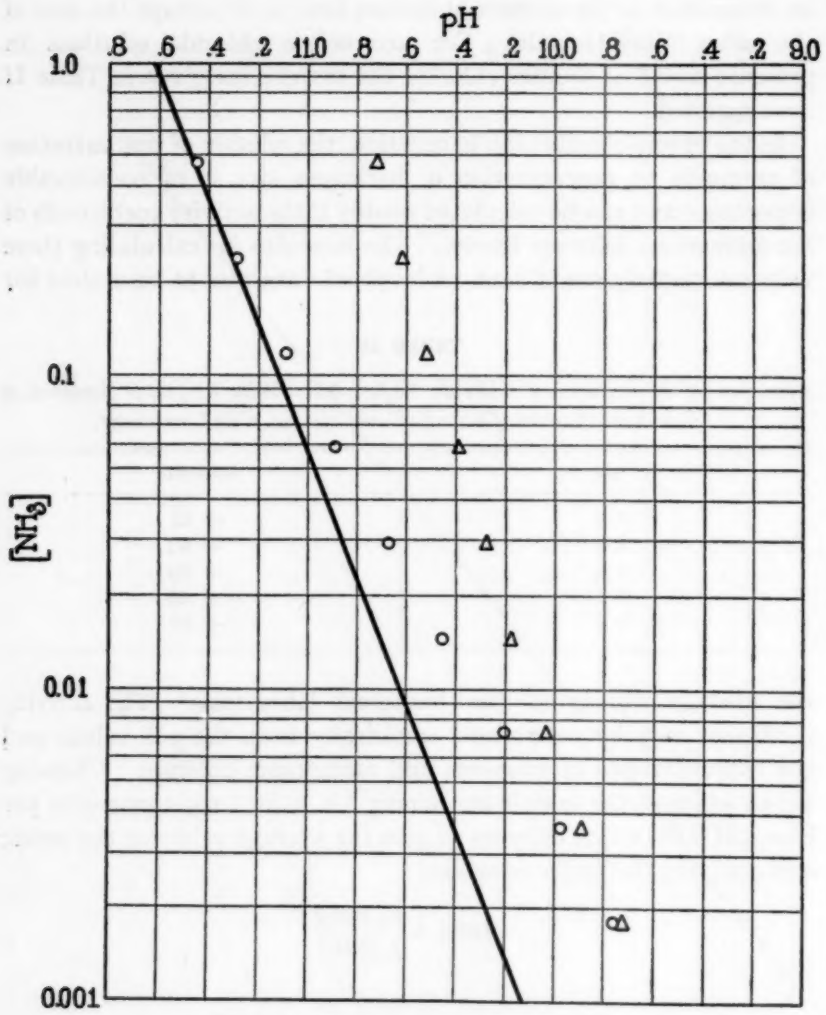

FIG. 3. Comparison of theoretical pH of ammonia in water with that observed with the glass electrode. O = Ammonia in water. Δ = Ammonia in 0.5 M sodium chloride solution.

calculated values from the simplified approximation formula of Bjerrum,[9] said not to be valid above about 0.1 M.

$$-\log f_a = \frac{26}{D} n^2 \sqrt[3]{c};$$

D is the dielectric constant of the solvent, and n the valence of the ions. As may be seen by reference to the table the agreement is not remarkable at any concentration, but is much better for 0.01 M and 0.1 M than for 0.25 M and 0.5 M solutions. The writer is inclined to think the empirical value for 0.5 M ammonium chloride the more accurate, and will use it in his calculations.

The measurement of pH of ammonia in water and in 0.5 M sodium chloride was less satisfactory. In the biological work involved, exclusion of carbon dioxide is not possible, so it was not attempted in this case. At the lower concentrations of ammonia the carbon dioxide introduced a serious error by the formation of an ammonium bicarbonate buffer system. At high concentrations of ammonia where the error due to carbon dioxide is negligible, there was no significant deviation from the calculated values in pure water, while in 0.5 M sodium chloride there was a large negative variation, 0.74 pH at pH 11.46. This variation decreased progressively as the ammonia was diluted and the manner of approach of the curves suggests its disappearance at about pH 9.0. For ammonia solutions from which sodium chloride is absent, the glass electrode may be used without appreciable error to pH 11.4 and perhaps higher. If sodium chloride is present a correction must be applied above pH 8.6, and will probably have to be determined anew for each electrode by the use of appropriate buffer solutions in which sodium ions are present in approximately the same concentration as in the unknown solution.

SUMMARY.

It is shown that the glass electrode may be used without appreciable error to measure pH of ammonia or ammonium chloride buffers, but that corrections must be applied above pH 8.6 if sodium ions are present in the unknown solution. Corrections are given for values from pH 8.6 to 9.4. A slight further modification of form of glass electrode used by previous workers is described.

[9] *Cf.* Michaelis, Hydrogen ion concentration, p. 121.

A METHOD FOR THE TITRATION OF COMPLEMENT.

By HARRY EAGLE.

(From the Department of Pathology and Bacteriology, Johns Hopkins Medical School, Baltimore.)

(Accepted for publication, March 16, 1929.)

A major difficulty in the quantitative study of complement is the accurate determination of the amount remaining after various manipulations. The method usually employed is to compare the minimal quantity of solution which will completely hemolyze a measured volume of sensitized cells with the corresponding quantity of the original serum. The method is cumbersome and time-consuming, necessitating multiple tests for each determination, and the results are only approximate.

Recently, Brooks[1] has suggested a method based on the colorimetric estimation of the proportion of cells hemolyzed by a given quantity of solution within an arbitrarily fixed time. By the use of a curve correlating the percentage of hemolysis with the quantity of complement he obtains results accurate to 1 per cent.

Needing a simple but accurate method adapted to making many determinations within a relatively short time, we have made use of the fact that the time required for hemolysis is a function of the quantity of complement. The data for a reference curve similar to Fig. 1 are first obtained with known quantities of complement, using a fixed amount of a standard sensitized cell suspension. The time required for an unknown solution to cause hemolysis *under the same conditions of temperature and volume* is then determined, and its complement content obtained by graphic interpolation on the time-complement curve (Fig. 1).[2]

[1] Brooks, S. C., *J. Med. Research*, 1919–20, xli, 399.

[2] Strictly speaking, the term minimal hemolytic quantity (unit) of complement has no significance unless qualified by the conditions under which the readings are made. In the experiment here reproduced, the unit of complement at 5 minutes is 0.026 cc., at 10 minutes, 0.0085 cc., and at 60 minutes < 0.004 cc. If it were not for an increasingly more rapid inactivation of complement as it is diluted, an infinitesimal quantity would suffice to cause hemolysis.

The following are the variables which must be controlled, and the values found most convenient:

Temperature.—37°C. If all determinations are made within an hour, it is possible to work at room temperature.

Final Volume.—2 cc.

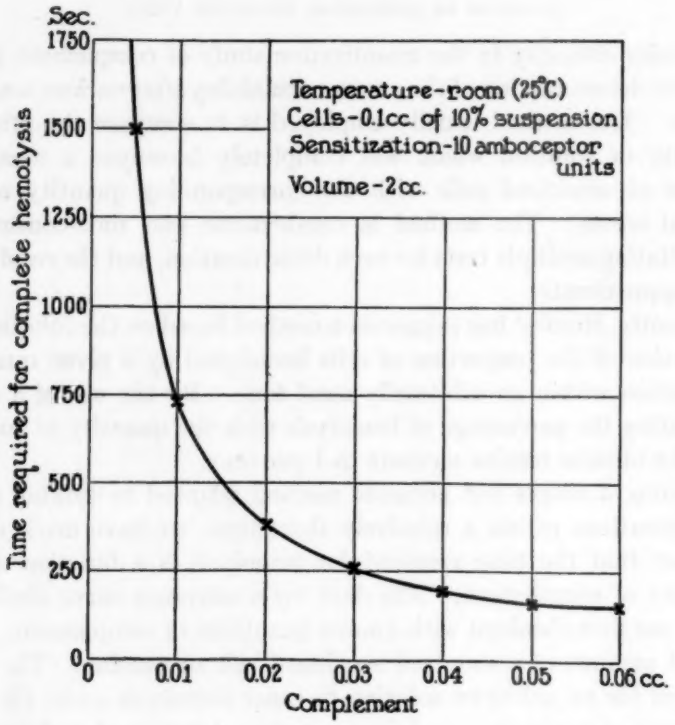

FIG. 1. Effect of quantity of complement upon the time required for complete hemolysis.

Test Suspension of Sensitized Cells.—*X* cc. of sheep's blood, citrated, are washed twice in 10 volumes of 0.85 per cent NaCl (at pH 7.4), and the sedimented cells resuspended to 10 times the original blood volume in NaCl. There are then added 15 sensitizing units of an immune rabbit serum. After 1 hour at room temperature (½ hour at 37°) the suspension is ready for use.

Order of Addition.—*X* cc. of the unknown solution (or of complement in obtaining data for the reference curve) are made up to a total volume of 1.9 cc. with

0.85 per cent NaCl, and the solution poured into a Wassermann tube containing 0.1 cc. of the sensitized cell suspension. The time required for *complete* hemolysis is noted, and the complement content determined in the manner described.

Provided only that the same test-cell suspension is used, under the same conditions, as many as 20 determinations can be made in an hour, 5 to 10 simultaneously.

It is obvious that any error in reading the endpoint is minimized in the steep descending portion of the curve, and exaggerated in the horizontal portion. Where feasible, it is therefore advisable to use that quantity of unknown solution which will give a hemolysis time in the zone of least experimental error.

THE MECHANISM OF COMPLEMENT FIXATION.

By HARRY EAGLE.

(From the Department of Pathology and Bacteriology, Johns Hopkins Medical School, Baltimore.)

(Accepted for publication, March 16, 1929.)

I.

Complement Fixation a Secondary Process in All the In Vitro Immune Reactions.

The *in vitro* immune reactions may be tabulated as follows:

1. Bacteria	+ Immune serum	→ Agglutination	
2. Soluble protein antigen (sheep serum, egg albumen, etc.)	+ Immune serum	→ Precipitation	
3. Red cells	+ Immune serum (amboceptor)	→ Sensitization	(± Agglutination)

It was shown by Bordet and Gengou (5) that the first of these reactions is accompanied by complement fixation, *i.e.*, if fresh guinea pig serum is present, it loses its ability to hemolyze sensitized red cells. Shortly thereafter, it was shown (16) that a similar inactivation of complement is obtained during the second of these reactions and, indeed, is a more sensitive indicator of the antigen-antibody interaction than the visible flocculation.

Whether a similar fixation occurs during the sensitization of red cells is as yet undetermined, due, of course, to the fact that hemolysis takes place. Thus, Liefmann and Cohn (20) state that although it is true that complement disappears, this inactivation is almost entirely a *result* of hemolysis, due to as yet unknown complicating factors, rather than a fixation by the sensitized cells. And although Michaelis and Skwirsky (22) showed that at an acid reaction (pH 5.3) which inhibits hemolysis, there is fixation (of the midpiece fraction), the experiments have been objected to on the ground that the high degree of sensitization necessary (25 amboceptor units) and the acid reaction are not duplicated under normal conditions.

The use of a simplified quantitative method of complement titration has made it possible to establish quite definitely that sensitized cells do fix complement; that there is a complete analogy to complement fixation by either agglutinated bacteria or the immune precipitate; and that this fixation, far from being a result of hemolysis, precedes it.

Methods.

1. Fixators.

(*a*) Sensitized cells.—Citrated sheep's blood is washed twice in 10 volumes of saline (NaCl N/7), resuspended to 10 times its original volume, and sensitized with 10 units of a rabbit anti-sheep serum.

(*b*) Immune precipitate.—Sheep plasma is incubated with the optimum quantity of a rabbit anti-sheep serum in 10 volumes of NaCl. The resulting precipitate, consisting almost entirely of globulins of the immune-serum, is washed twice in 10 volumes of NaCl, and resuspended to 15 times its original volume. As little of 0.1 cc. of this suspension, representing 0.02 cc. of the serum used, fixes 0.4 cc. complement (20 units) almost completely.

(*c*) Agglutinated bacteria.—*B. typhosus*, washed free of the broth medium, incubated with a rabbit anti-serum. The agglutinated bacteria, washed 3 times in 10 volumes of NaCl, are resuspended to 15 times their own volume in NaCl.

2. Complement.—Guinea pig serum.

3. Titration of residual complement.

The method used is described in detail elsewhere (12), and is based on the fact that the time required to hemolyze a fixed volume of a standard sensitized cell suspension, is, under constant conditions of temperature and volume, a function of the quantity of complement. For the determinations, the fluid, freed from the fixing substance by strong centrifugation, is poured into a tube containing 0.2 cc. of a 10 per cent sheep cell suspension sensitized with 10 units of a hemolytic serum, and the time required for complete hemolysis noted.

Protocol 1. The Adsorption of Complement by Sensitized Cells.—To a series of tubes containing X cc. of sensitized cells are added 0.04 cc. of complement and NaCl (N/7) to 2 cc., at varying times before beginning of centrifugation. The tubes are placed in the centrifuge as they are prepared, and a minimum time allowed to elapse between the preparation of last tube and beginning of centrifugation (2500 r.p.m. for 1 minute). The clear supernatant fluid is poured into a tube containing 0.1 cc. of sensitized cells, and the time necessary for complete hemolysis noted. By a method already described (12), the amount of complement present in the supernatant may be calculated from the velocity of hemolysis. This quantity subtracted from that originally present, gives that adsorbed by the sensitized cells.

The time X in the figures corresponds to the adsorption which takes place

during centrifugation. The sedimentation of the strongly sensitized cells is so rapid that X is certainly less than 20 seconds, and in any one experiment, is obviously the same in all the tubes.

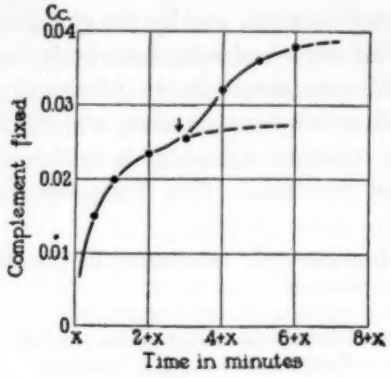

FIG. 1.

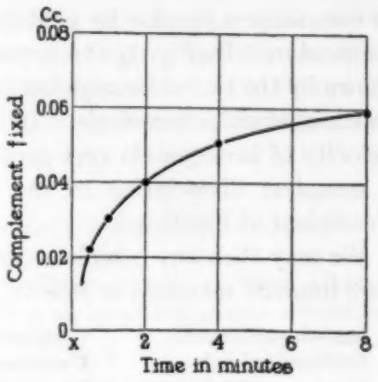

FIG. 2.

FIG. 1. Complement fixation by suspension of sensitized cells.

FIG. 2. Complement fixation by the precipitate formed in an antigen antibody reaction.

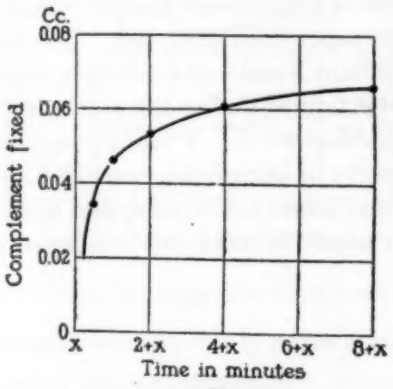

FIG. 3. Complement fixation by a suspension of agglutinated bacteria.

The inflection in the curve, signifying a sudden increase of fixation, is coincident with the beginning of hemolysis and explains the experimental data of Liefmann and Cohn. Its significance will be discussed

in a later paper. The dotted lines indicate the probable course if it were not for this confusing secondary fixation.

For comparison, there are introduced similar curves for the velocity of complement fixation by agglutinated bacteria, and by the globulin suspension. In Fig. 1, the arrow indicates *beginning* hemolysis, as shown by the tint of hemoglobin in the supernatant fluid. Obviously, fixation precedes hemolysis. Indeed, at low temperatures, where the velocity of hemolysis is very greatly retarded, it is possible to obtain a complete dissociation of the two reactions. (See Temperature Coefficient of Fixation.)

We may therefore complete the diagrammatic tabulation of the *in vitro* immune reactions as follows:

1. Agglutinated bacteria	+ Complement → Complement fixation	± Bacteriolysis
2. Sensitized red cells	+ Complement → Complement fixation	± Hemolysis
3. Immune precipitate	+ Complement → Complement fixation	± Anaphylatoxin
4. Beef heart extract-syphilitic serum complex	+ Complement → Complement fixation	

Although most observers have concerned themselves with only the last two of these reactions, even a cursory survey of the literature relating to the theories of complement fixation would go far beyond the scope of the present paper (17), (27), (30). The recent tendency has been to regard Reactions 3 and 4 as a non-specific physical process, in which the aggregates formed during the antecedent antigen-antibody reaction adsorb complement, *i.e.*, a labile component of fresh serum which has the property of hemolyzing sensitized cells.

The following experiments are in full accord with such a hypothesis, and extend it to include all of the *in vitro* immune reactions listed above.

II.

The Kinetics of Complement Fixation.

$$\text{Antigen} + \text{Antibody} \rightarrow \left.\begin{matrix}\text{Agglutinated bacteria}\\ \text{Sensitized cells}\\ \text{Precipitate}\end{matrix}\right\} \textit{i.e.}, \text{ Immune-complex}$$

$$\text{Immune-complex} + \text{Complement} \rightarrow \text{Complement fixation}$$

It is apparent that in order to arrive at the kinetics of the second reaction, it is essential to eliminate the time and quantity factors introduced by the first. Such a reaction as the Wassermann, in which

antigen, "antibody" and complement are added simultaneously, is of no value in arriving at the physical constants of complement fixation. No matter how exactly measured, the results obtained apply to neither of the two reactions individually, but to a complex of two consecutive reactions of quite different character. For this reason, in the following experiments the fixing agent used was an antigen-antibody mixture which had been allowed to come to equilibrium before the addition of complement.

A. Effect of Reactants upon the Velocity of Fixation.

Protocol 2.—To X cc. of the standard globulin suspension are added 1.0 cc. of 1/10 complement at the times noted in Table I, the tubes being placed in centrifuge

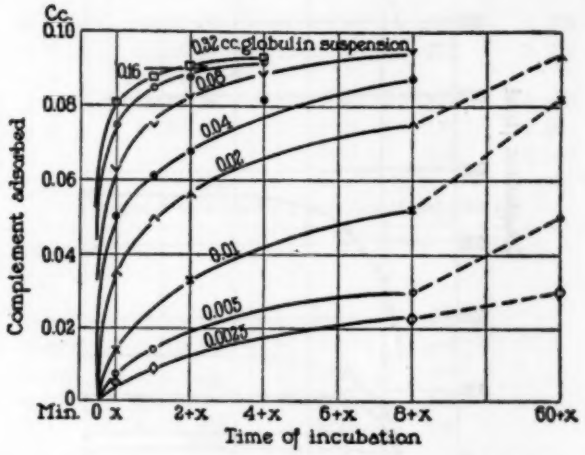

FIG. 4. Effect of globulin concentration upon velocity and degree of complement fixation.

cups as they are prepared. At the indicated time, centrifugation is begun, 60 seconds at 2500 r.p.m. sufficing to throw down practically all the particles of denatured globulin.

The supernatant fluid is poured directly into a tube containing the test cells (see page 826) and the time required for hemolysis noted. The quantity of free complement corresponding to this hemolysis time, subtracted from the original quantity added, gives that fixed by the globulin suspension (Fig. 4).

In the curves, the time X corresponds to the fixation which occurs during cen-

trifugation before the globulin is completely sedimented. In any one experiment, this is obviously the same for all the tubes, and is probably less than 20 seconds.

If now, the quantity of globulin is kept constant, and that of complement varied, we obtain the series of curves given in Fig. 5.

The essential identity of all the fixation reactions is again illustrated by the fact if one uses sensitized cells or agglutinated bacteria as the fixing agent instead of the globulin suspension, exactly similar sets of curves are obtained (Figs. 6, 7, 8).

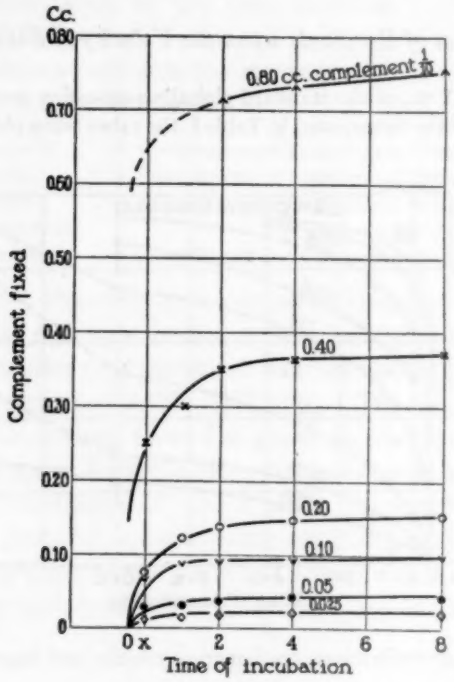

FIG. 5. Effect of (complement) upon velocity and degree of complement fixation by globulin suspension.

In all the experiments, increases of either of the two reactants has the same effect: more complement is fixed, and the reaction accelerated. *Qualitatively*, therefore, the reaction proceeds according to the mass action law.

$$\frac{\text{[Complement]} \times \text{[Antigen-antibody complex]}}{\text{[Complement fixed, } i.e.\text{, Antigen-antibody-complement]}} = K$$

and the velocity of reaction

$$\propto [\text{Complement}]$$
$$\propto [\text{Antigen-antibody complex}]$$

The steep initial rise, and the rapid change in slope as the equilibrium value is approached, are difficult to interpret; but it is quite significant that similar curves have been obtained for the most diverse kinds of

TABLE I.

Data Used in Plotting Curve 0.02 of Fig. 4.

Time of addition of globulin to complement	Minutes before centrifugation	Time at which supernatant is added to test cells	Time at which hemolysis of test cells is complete	Seconds required for complete hemolysis	Complement remaining free in solution	Complement adsorbed by globulin
					cc.	*cc.*
10:15	60 + x	11:18:50	11:41:50	1380	0.006	0.094
11:07	8 + x	11:18:45	11:23:15	270	0.024	0.076
11:11	4 + x	Tube broken				
11:13	2 + x	11:18:35	11:21:50	195	0.043	0.057
11:14	1 + x	11:18:30	11:21:20	170	0.05	0.05
11:15	x	11:18:25	11:20:55	150	0.063	0.037
Controls* with known quantities of complement.						
	Complement					
	cc.					
	0.0062	11:18:20	11:40:15	1320		
	0.0125	11:18:15	11:27:35	560		
	0.025	11:18:10	11:22:30	260		
	0.05	11:18:05	11:20:55	170		
	0.1	11:18	11:19:40	100		

* By plotting the time required for increasing quantities of complement to cause hemolysis (control tubes) one obtains the reference curve used in arriving at the values for residual complement given in Column 6. These control tubes must be duplicated with each similar experiment, using the same cell suspension under the same conditions.

adsorption processes, with a similar acceleration produced by increasing the concentration of either the adsorbent or adsorbate (2), (4), (21).

B. Temperature Coefficient.

Using a constant quantity of fixing suspension and complement, the

velocity of fixation was determined at various temperatures between 5 and 40°C. The results of three such experiments are summarized in Figs. 9, 10, 11, using the globulin suspension, agglutinated bacteria, and sensitized cells respectively, as the fixing agent.

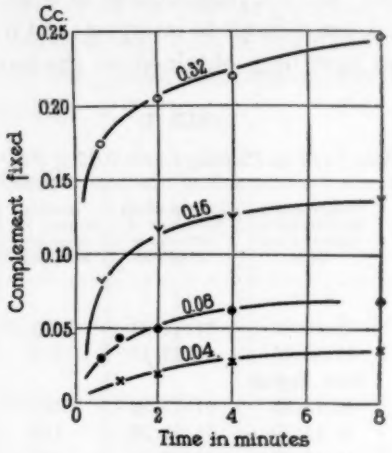

FIG. 6. Effect of (complement) upon complement fixation by sensitized bacteria.

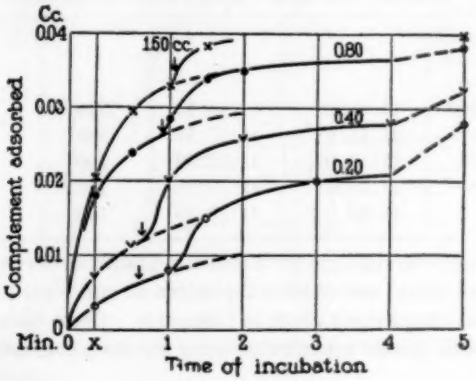

FIG. 7. Effect of (sensitized cells) upon velocity and degree of complement adsorption.

The similarity of the curves is further evidence of the essential identity of all the complement fixation reactions. It is apparent that the absolute magnitude of the temperature coefficient will depend entirely on its definition: if the amount fixed at equilibrium is taken as the

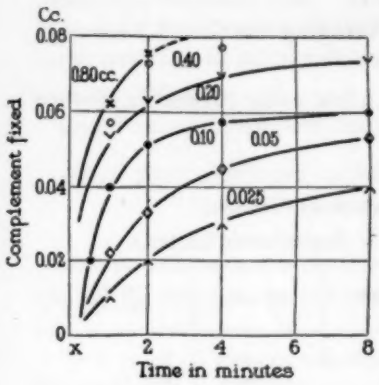

FIG. 8.

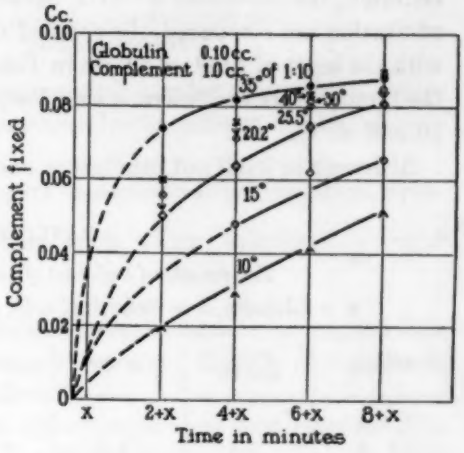

FIG. 9.

FIG. 8. Effect of (bacteria) upon complement fixation by a suspension of agglutinated bacteria.

FIG. 9. Effect of temperature upon complement fixation by globulin suspension.

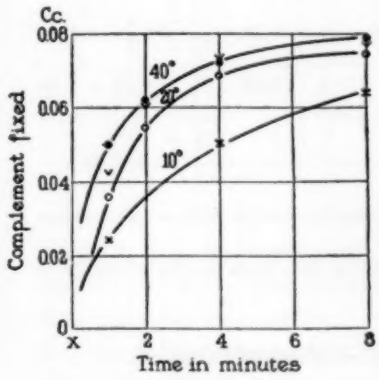

FIG. 10.

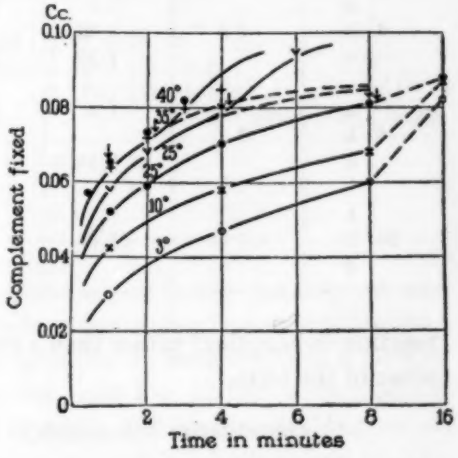

FIG. 11.

FIG. 10. Effect of temperature upon complement fixation by agglutinated bacteria.

FIG. 11. Effect of temperature upon complement fixation by sensitized cells.

criterion, the coefficient is <1.1, between 5 and 40°C.; if the velocities of fixation are compared, the value of the coefficient decreases steadily with the lapse of time, as shown in Table II. But, however expressed, the temperature coefficient is very low, averaging less than 1.3 between 10 and 40°C.

Although in itself not conclusive, such a low value suggests a surface

TABLE II.

Temperature Coefficient of Complement Fixation.

a = Globulin, b = Sensitized cells, c = Agglutinated bacteria.

Minutes		5°–10° (per 10)	10°–20°	20°–30°	30°–40°	10°–40° (per 10)
	a					
1	b	2.5	1.3	1.3	1.05	$\sqrt[3]{2.2} = 1.30$
	c		1.5			$\sqrt[3]{2.1} = 1.28$
	a		2.25	1.12	1.07	$\sqrt[3]{3.3} = 1.49$
2	b	2.4	1.3	1.1	1.06	$\sqrt[3]{1.9} = 1.24$
	c		1.5	1.17	1.05	$\sqrt[3]{1.8} = 1.22$
	a		2.1	1.13	1.05	$\sqrt[3]{2.7} = 1.39$
4	b	2.3	1.28			$\sqrt[3]{1.6} = 1.19$
	c		1.36	1.1	1.05	$\sqrt[3]{1.4} = 1.12$
	a		1.6	1.06	1.05	$\sqrt[3]{1.8} = 1.22$
8	b	2.2				
	c		1.16		1.05	$\sqrt[3]{1.25} = 1.08$
	a					
16	b	<1.05	<1.05			<1.05
	c					

reaction (adsorption) rather than a chemical reaction in the restricted sense of the term.

C. Quantitative Relationships in Complement Fixation.

If, varying the quantities of complement and fixing suspension, we determine, not, as heretofore, the velocity, but the conditions at equilibrium, we obtain results similar to those summarized in Table III, in which each horizontal row represents an individual experiment. Obviously, there is no stoichiometric relationship in the sense that

$$n_1 \text{ Complement} + n_2 \text{ Antigen-antibody} \rightarrow n_3 \text{ Antigen-antibody-complement (complement fixed)}$$

Nor does the degree of fixation correspond to a distribution of complement between fixing agent and solution in the ratio of its solubility in the two phases.

Qualitatively, however, the results do conform to the empirical equation of Freundlich for adsorption from dilute solutions: $\frac{x}{m} = Kc^{\frac{1}{n}}$

TABLE III.

Quantitative Relationships in Complement Fixation by I Globulin, II Agglutinated Bacteria.

To X cc. of (I) globulin suspension, (II) agglutinated bacteria, are added Y cc. of complement, and the total volume brought to 2 cc. with NaCl N/7. The figures given in the body of the table represent the complement fixed after 2 hours at room temperature (25°C.).

	Complement	Fixing suspension								
		0.64 cc.	0.32 cc.	0.16 cc.	0.08 cc.	0.04 cc.	0.02 cc.	0.1 cc.	0.05 cc.	0.025 cc.
	cc.									
I	1. 1.28	1.273	1.26	1.24	1.12	0.96				
	2. 0.32			0.315	0.309	0.293	0.24			
	3. 0.16				0.157	0.1535	0.142	0.1		
	4. 0.08						0.077	0.071	0.05	0.028
II	5. 1.28	1.26	1.224	1.16	0.96	0.58				
	6. 0.64		0.623	0.6	0.534	0.44				
	7. 0.32				0.305	0.289	0.252	0.16		

where X is the total quantity adsorbed, m is the quantity of adsorbent, c is the amount of adsorbate remaining free at equilibrium, and K and n are constants, characteristic for each reaction, n always being greater than unity. It is seen from Fig. 12 that both types of fixation follow such a curve very closely. The n value of all but one of the curves fitted to the experiments in Table III is very close to 1.5.

If the Freundlich equation were an exact expression of the reaction, then the logarithms of $\frac{x}{m}$ plotted against the logarithms of c, would

fall along a straight line, whose slope is determined by the value of n. It has been shown for many types of adsorption that this is true only at low values of c; that as the amount of free adsorbate increases, the curve gradually bends over, becoming finally almost parallel to the

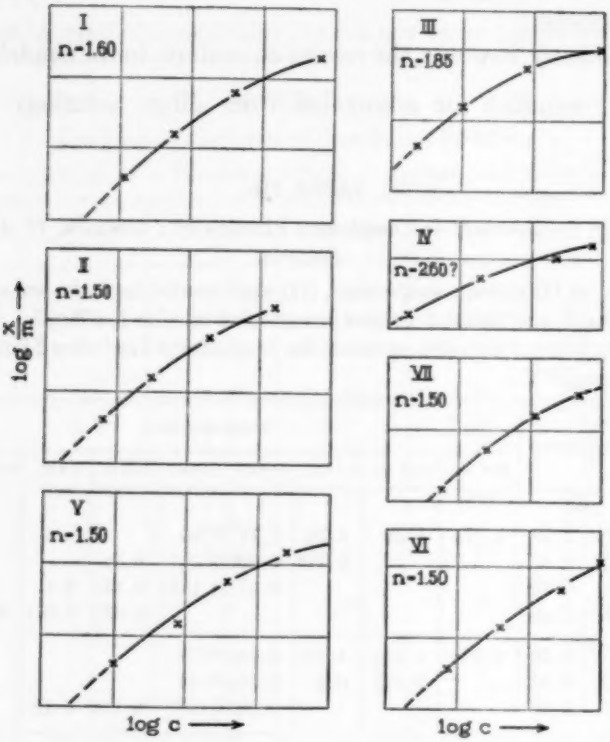

FIG. 12. Deviation of complement fixation from the adsorption isotherm of Freundlich.

$$\log \frac{x}{m} = \left(\frac{1}{n} \times \log c\right) + k'$$

c axis. The complement fixation reaction shows the same deviation from the Freundlich equation (Fig. 12).

D. Summary.

1) Since complement fixation has a very low temperature coefficient; 2) since its velocity does not conform to that of a bimolecular reaction,

but resembles that of adsorption reactions in general; 3) since the quantitative relationships between complement and fixing agent are not stoichiometric, but conform to those encountered in all adsorption reactions, even though as yet unexplained; and 4) since the reaction obviously takes place at the surface of a heterogeneous phase, we may characterize it as an adsorption process.

The experiments already presented show clearly that all types of complement fixation, whether by the immune globulin suspension, sensitized cells, or agglutinated bacteria, are fundamentally the same

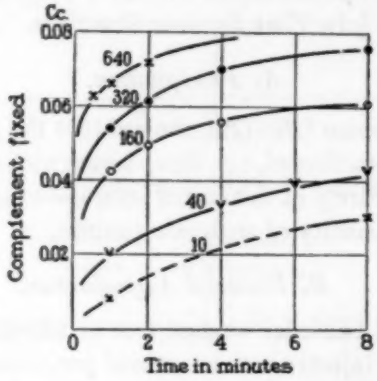

FIG. 13. Effect of immune serum upon complement fixation by agglutinated bacteria. Numbers on the curves indicate relative quantity of immune serum used.

reaction. The adsorbate is always, of course, "complement;" it remains to characterize the adsorbent.

In the precipitation reaction the adsorbent is the precipitate itself (7), (9), (10), (11). The question is, what is the physical basis for the complement fixing property of the antigenic cell after it has been incubated with immune serum?

The experiment summarized in Fig. 13 proves that the complement fixation obtained with bacteria (and presumably[1] red cells) is due entirely to some alteration in the cell surface caused by the immune serum, which is proportional to the degree of sensitization. The most logical hypothesis is that during sensitization there is an aggregation

[1] This has since been shown to obtain for red cells also (13).

of immune-serum globulin upon the surface of the cells, identical with that formed during the precipitation reaction: and that complement fixation in *all* the immune reactions is due to an adsorption by these aggregates.[2]

The following data already in the literature prove the formation of just such a surface film of immune serum protein during cellular sensitization.

III.

Aggregation of Immune Serum Protein as a Common Factor in all the In Vitro Immune Reactions.

A. Precipitation.

Welsh and Chapman (28), (29), showed that the precipitate which is formed by the interaction of, *e.g.*, sheep serum with a rabbit anti-serum consists almost entirely of denatured immune-serum globulin, with a relatively small quantity of antigenic protein.

B. Bacterial Agglutination.

1. Agglutinated bacteria, washed free of excess serum, will cause anaphylaxis when injected into an animal previously sensitized to the species of serum used (6).

2. Normal bacteria have a negative change, reversed only by trivalent cations (24), or at a very acid reaction (pH 3.5 (23)). But as the degree of sensitization is increased, their cataphoretic isoelectric point gradually approaches pH 4.7 ((23) horse serum), and, according to Shibley, becomes identical with that of particles of heat-denatured serum globulin ((25) rabbit serum). A phenomenon, noted long before these recent findings, is a direct corollary: specific agglutination is enhanced by cations at serum reaction (when the change in the organisms is negative), and by anions in acid reaction (when the change in the organisms, *determined by the serum globulin surface* is positive) (3), (19), (26).

3. Normal bacteria agglutinate in acid reaction, the optimum range being, to a certain extent, characteristic for each species. When

[2] This aggregation of immune-serum protein upon the surface of the *individual* cell is not to be confused with the subsequent agglutination of many cells.

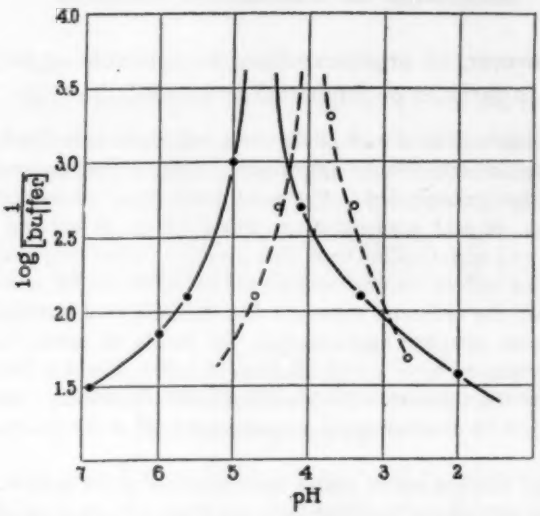

FIG. 14. Effect of pH upon the agglutination of sensitized and normal pneumococci by electrolyte.

———— = Sensitized.
---------- = Normal.

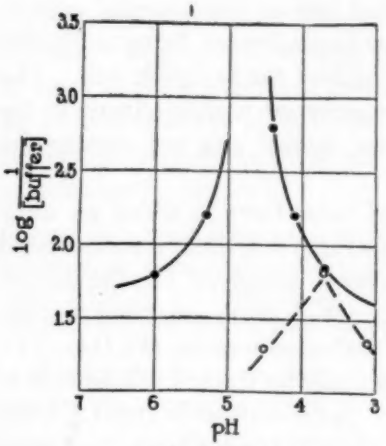

FIG. 15.

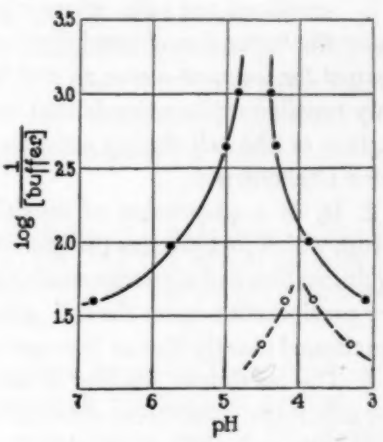

FIG. 16.

FIG. 15. The effect of pH upon the agglutination of sensitized (————) and normal (----------) *B. typhosus* by electrolyte.

FIG. 16. The effect of pH upon the agglutination of *B. pertussis* by electrolyte.

———— = Agglutinated with immune serum.
---------- = Normal.

sensitized, however, all organisms have an optimum agglutination at the same pH as particles of denatured serum globulin (Figs. 14, 15, 16).

Protocol 3.—Suspensions of each of the three organisms named below are incubated with approximately twenty agglutinating units of immune serum, washed 3 times in 20 cc. and resuspended to 50 times the volume of the sedimented cells in H_2O. To 0.2 cc. of each suspension are added 0.8 cc. of varying dilutions of acetate buffers, and agglutination read after 2 hours at room temperature.

In the following figures, the abscissae are the pH of the buffer when N/10 with respect to NaAc; the ordinates represent the minimal concentration of buffer[3] necessary to cause complete agglutination. It should be noted that the peaks of the curves correspond to very high dilutions of buffer, at which the pH can only be very roughly approximate. As determined colorimetrically, this correction increases steadily with dilution, and is as much as 0.6 pH at the peaks of the curves (buffer N/4000).

It is seen that immune serum makes the organisms much more susceptible to agglutination by electrolytes, and that there is a sharply defined optimum reaction for agglutination, *the same for all three organisms*, at about pH 5.0 (making the approximate correction).

C. Red Cell Sensitization.

1. Sensitized red cells, when injected into an experimental animal, cause the formation of precipitating and complement-fixing antibodies *against the immune-serum* as well as against the antigenic cell. The only possible explanation is that immune serum protein adheres to the surface of the cell during sensitization, normal sera not causing the same phenomenon.

2. If, to a suspension of sensitized cells, there is added an antiserum which precipitates the immune serum used, there is an increased agglutination and a greater susceptibility to hemolysis, *i.e.*, the secondary precipitation upon the cell surface, which could only be of protein, functioned exactly like an increase of amboceptor serum (1), (14), (15).

3. The maximum binding of amboceptor by the red cell, as well as the minimum subsequent dissociation (in saccharose) is at pH 5.3, the isoelectric point of serum globulin, where it is most labile, and most readily thrown out of solution (8), (18).

4. The optimum range for the agglutination of sensitized cells is around pH 5.3 (7).

[3] (NaAc) is taken as (buffer), the ionization of the HAc being negligible in comparison.

Conclusion.

Knowing that the washed suspension of denatured immune-serum globulins, formed during the precipitation reaction fixes complement powerfully; and that a similar aggregation of immune serum globulin upon the surface of the antigenic cell accompanies bacterial agglutination and red cell sensitization, we may conclude that it is this surface film of protein which determines complement fixation by adsorbing the labile complement adsorbate.

IV.

The Nature of the Attractive Force Between Complement and the Adsorbing Aggregate.

There is one peculiarity of the complement fixation reaction which is not explained by the concept just outlined. Whatever the forces involved, adsorption is by definition a surface reaction. How are we to explain the fact that the precipitate formed in an immune reaction has at least 20 times as great an avidity for complement as a suspension of, *e.g.*, Kaolin, or normal bacteria, or carbon particles of approximately the same microscopic size as the elements of the precipitate? Why should heating this globulin suspension at 90°C. for 30 minutes destroy its fixing properties, so that it becomes as inefficient an adsorbent as the particles just cited? Why does a suspension of heat-denatured normal serum globulin not possess the same avidity for complement? (Fig. 17.)

Protocol 4.—Each of the suspensions listed in Fig. 17 is made up to 15 times the volume of the sedimented particles in NaCl N/7. To *X* cc. of the suspension are added 0.8 cc. of 1/10 complement and NaCl to 2 cc., and residual complement determined after 2 hours at room temperature.

These questions can only be answered when we know more concerning the essential nature of adsorption reactions in general. If adsorption is determined solely by the *physical state* of the *surface*, irrespective of its chemical character, then we may conceive of the precipitate formed in the immune reaction as possessing, *e.g.*, a submicroscopic honeycomb-structure allowing for an enormous increase in surface and ability to adsorb: heat would destroy this "inner surface" so that the particles resembled Kaolin, normal bacteria, etc., in their surface (adsorption) properties.

The recent tendency, however, is to regard adsorption as due to the usual chemical forces of attraction operating at the surface of a heterogeneous phase. So considered, some thermolabile substance would be present in the antigen-antibody complex with a specific chemical affinity for complement. Although it is difficult to explain the adsorption of complement, however weak, by such an inert substance as Kaolin on this basis, the question must be left open for further investigation.

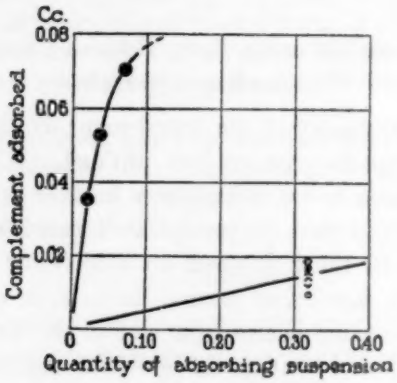

FIG. 17. The adsorptive affinity properties of various suspensions for complement.

- ● Suspension of specific precipitate. (Sheep *vs.* antisheep serum.)
- ◇ Specific precipitate heated at 90° ten minutes.
- . Heat Denatured egg albumen.
- × Heat Denatured serum globulin.
- ∧ Heat Denatured serum albumen.
- ∨ Kaolin.
- o Normal *killed* bacteria.

SUMMARY.

1. Complement fixation is obtained in every antigen-antibody reaction involving the presence or formation of a heterogeneous phase (red cells, bacteria, precipitate).

2. The physical constants of fixation (temperature coefficient, velocity, quantitative relationships between the reactants) are those

commonly associated with adsorption processes, and are the same in the three types of fixation studied.

3. All the *in vitro* immune reactions involve an aggregation of immune-serum globulins upon the surface of the antigen. It has been shown that the "fixation" of complement is an adsorption by the aggregates so formed; whether these aggregates are visible as a flocculent precipitate (*e.g.*, sheep serum *vs.* anti-serum) or concentrated as a surface film on a cellular antigen (sensitized cells; agglutinated bacteria), the reaction is fundamentally the same.

4. As yet, it is unknown whether this adsorption is determined by the physical state of the precipitate, and thus, differs only quantitatively from that by Kaolin, charcoal, normal bacteria, heat-denatured proteins, etc.; or whether the comparatively enormous avidity of these aggregates for complement is due to a specific chemical affinity.

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MECHANISM OF HEMOLYSIS BY COMPLEMENT.

I. Complement Fixation as an Essential Preliminary to Hemolysis.

By HARRY EAGLE and GEORGE BREWER.

(From the Department of Pediatrics and the Department of Pathology and Bacteriology, Johns Hopkins Medical School, Baltimore.)

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Despite an enormous literature which has accumulated since the discovery of the bactericidal, bacteriolytic, and hemolytic properties of fresh serum, the terms alexin and complement still denote unexplained properties of serum rather than a chemical entity. The hemolysis of sensitized cells, as the most clearly defined of these immunologic properties, has been that most studied, and many theories have been suggested as to its mechanism. The analogy drawn by Noguchi (16) between complement and certain hemolytic lipoids has been found by Liefmann and Cohn (10) to be unwarranted. Dick (3), finding increased amino-acids after hemolysis, was inclined to identify complement as a proteolytic ferment; Olsen and Goette find a suggestive analogy between complement and serum lipase (18); while, more recently, it has been interpreted as an intracellular catalyst allowing the release of hemoglobin by the amboceptor ferment (9). The statement, which we have since found to be erroneous, that complement is not consumed during hemolysis (1), (11), seemed to support the theory of its enzymatic (catalytic) action. At best, the evidence is inconclusive, and the rôle of the preliminary sensitization with immune serum remains unexplained.

I.

Complement Fixation by Sensitized Cells.

It has already been shown (5) that sensitized cells "fix" complement, and that this fixation is exactly similar to that by agglutinated bacteria, or by the precipitate formed when, *e.g.*, sheep serum is added

to a rabbit anti-sheep serum. Because this fixation is obviously a surface reaction, with a very low temperature coefficient, and because

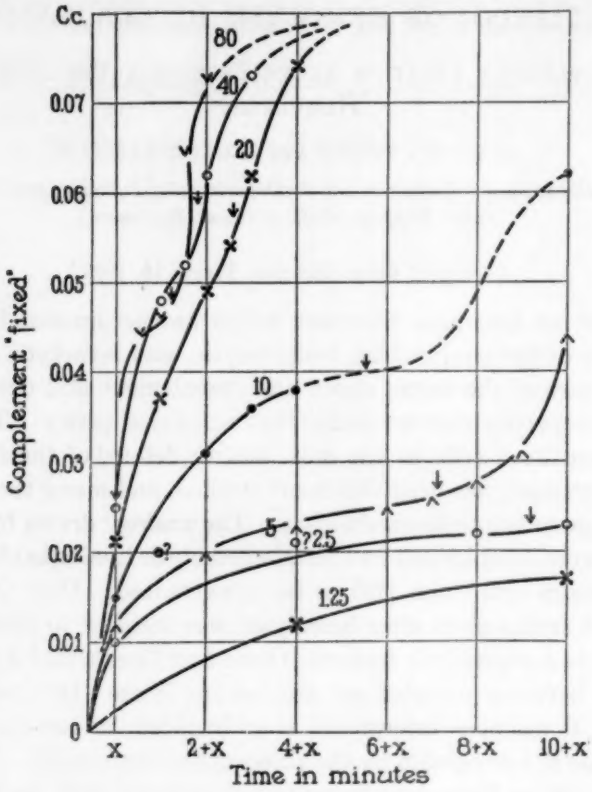

FIG. 1. Effect of degree of sensitization upon complement adsorption by sensitized cells.

To 3.5 cc. of a washed 10 per cent suspension of cells are added immune serum and NaCl N/7 to a total volume of 4 cc. The numbers on the curves are 1000 times the volume of immune serum used in each experiment. After 2 hours at room temperature, 0.4 cc. of the sensitized cell suspension is added to each of 10 tubes and the supernatant fluid removed by *slow* centrifugation. 0.08 cc. complement in 2 cc. NaCl N/7 is added to the sedimented cells at X minutes before centrifugation. This second centrifugation is performed at high speed, for exactly 1 minute, the clear supernatant carefully decanted into a tube containing 0.1 cc. of a standard test suspension, and the residual complement determined by a method already described (4) from the time required for complete hemolysis.

the quantitative relationships conform with reasonable accuracy to the empirical adsorption isotherm of Freundlich, it has been characterized as an adsorption process. The adsorbate is, of course, the labile substance in fresh serum called complement; and it was suggested that the adsorbent is *the same in all the in vitro immune reactions*: the immune-serum protein which adheres on the surface of the antigen during sensitization, agglutination, or precipitation. In the latter, it is visible as a precipitate consisting almost entirely of immune-

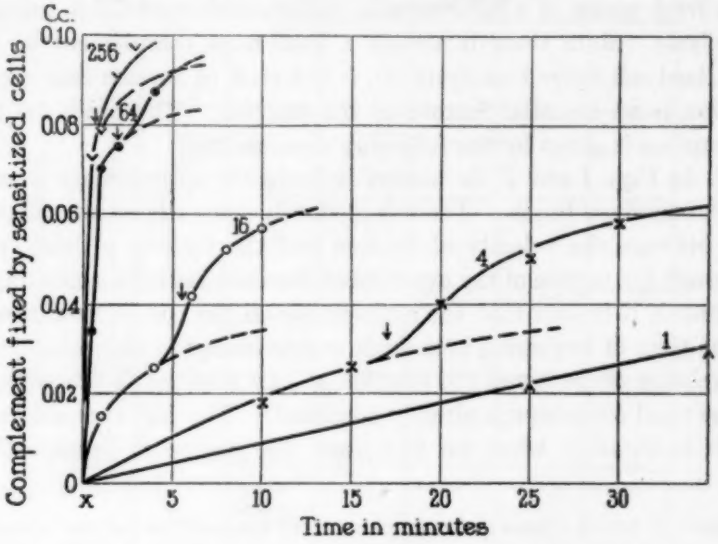

FIG. 2. Effect of degree of sensitization upon complement fixation.

serum globulins, but containing also antigenic protein. In the former it can be demonstrated immunologically, and by changes in the surface properties of the antigenic cell (cataphoresis; agglutination; effect of electrolytes).

The following experiment indicates that this hypothesis is fundamentally correct: using a constant quantity of cells, the velocity and degree of complement fixation is seen to increase enormously with the degree of sensitization, *i.e.*, with the amount of immune serum protein taken up by the cell[1] (Figs. 1 and 2).

[1] Whether or not the immune body is protein, it is always associated with the serum globulin. Increased sensitization results, not only in greater fixation of

It remains to show that this fixation is an essential preliminary to hemolysis; that complement is entirely inactive until it has been so adsorbed by the sensitized cell.

II.

The Adsorption of Complement by the Sensitized Cell as a Determining Factor in Hemolysis.

By definition, complement fixation is the removal (adsorption) from fresh serum of a substance (or substances) essential in immune hemolysis. Since there is always a fixation of complement by the sensitized cell *before* hemolysis (5), it is logical to assume that such a fixation is an essential feature of the reaction. The proof for this assumption is given by the following experiments.

(*a*) In Figs. 1 and 2 the arrows indicate the approximate time at which hemolysis began. There is obviously some significant relationship between the velocity of fixation and the velocity of hemolysis. Although the nature of the experiment does not justify a quantitative treatment, it is seen that the areas under all the curves from time 0 to the time of beginning hemolysis is *approximately* a constant, *i.e.*, the velocity of the hemolysis reaction at any moment is proportional to the total complement already adsorbed.[2] The fact that the same result is obtained when we vary, not the degree of sensitization,

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complement, but in a more and more pronounced tendency for the cell to assume the surface characteristics of a particle of denatured globulin.

[2] Let $C_A = f(t)$ = Complement adsorbed by cell

$\frac{dh}{dt}$ = Velocity of the complement-cell reaction, and

H = Amount of this reaction required for hemolysis, obviously a constant for any one suspension

t_h = Time required for hemolysis

Then $\int_0^{t_h} C_A dt = k = KH = K\int_0^{t_h} \frac{dh}{dt}$ in all the curves.

And $\frac{dh}{dt} = KC_A$: the velocity of hemolysis at any time is proportional to the total complement already adsorbed. This statement is an approximation only of the true relationships, and becomes quite inaccurate at high concentrations of complement. The kinetics of hemolysis will be discussed in a later paper.

but the amount of complement (Fig. 3) indicates a true causal relationship.

(*b*) The marked influence of electrolyte concentration and the reaction of the solution upon hemolysis is due to the concomitant inhibition of complement fixation.

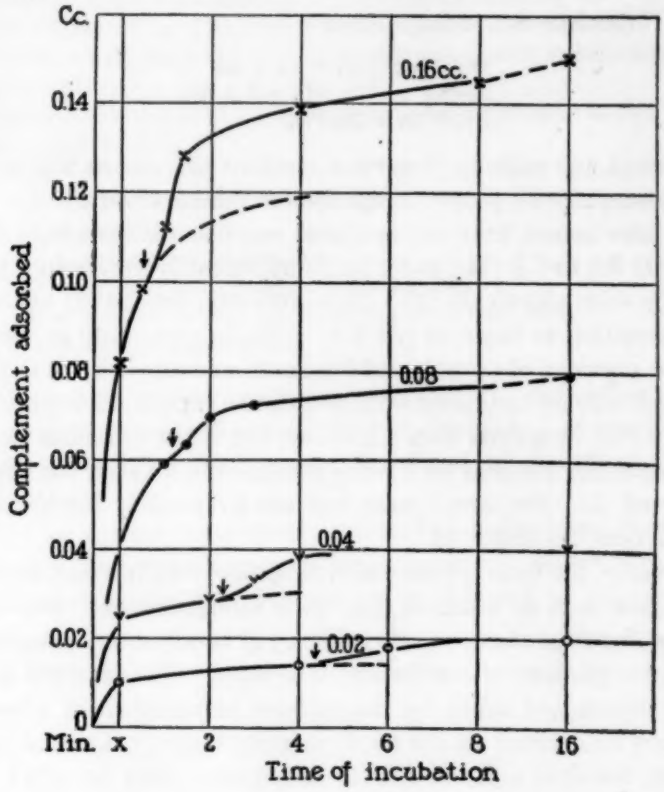

FIG. 3. Effect of (complement) upon velocity and degree of complement adsorption.

One of the earliest observations concerning complement was that it is destroyed by dilution with glucose or water, and that hemolysis does not occur in the absence of electrolytes (7), (13), (19). Manwaring (12) noted the inhibitory effect of electrolytes in excess, and suggested

as its cause the formation of a loosely bound salt-complement compound, from which active complement could be liberated by precipitation of the salt. Noguchi (17) commented on the strong inhibition by salts of Ca or Ba. More recently Wright and MacCallum (23) have tested the effects of many salts in hemolysis, and have arranged the ions in the order of their inhibitory activity in a series analogous to the Hofmeister ion series. Thus

$$K > Na > NH_4 > Ca > Ba$$
$$Acetate > Cl > NO_3 > I > SO_4$$
$$Na_2HPO_4 > Na_3PO_4$$

No attempt was made to preserve a constant pH, and as will be seen, this is essential for a proper comparison of the ion effects.

It is also known that the optimum reaction for hemolysis lies at about pH 7.0 to 7.5 (14), and that complement is irreversibly inactivated by acids and alkalis (6). More recently, Brooks (2) has found the destruction to begin at pH 5.3. This is apparently at variance with the previous observation of Michaelis and Skwirsky (14) that if sensitized cells are incubated with complement at pH ±5.6 (phosphate buffers 1/16) hemolysis does not occur; but if the optimum reaction is subsequently restored by adding alkaline phosphates, the cells are hemolyzed, *i.e.*, the complement was not irreversibly inactivated in acid reaction, but inhibited.

Obviously, the facts require re-investigation. Of interest, however, are not the data so obtained, but their interpretation. For *it will be shown that these changes in the efficiency of hemolysis are due to variations in the efficiency of complement adsorption.* The degree of hemolysis is determined solely by the amount of complement adsorbed, and bears no relation to the total quantity added. And failing adsorption, multiple units of active complement have no effect upon sensitized cells.

1. Methods.

(*a*) *Hemolysis.*—The rabbit anti-sheep cell system was used throughout. Large quantities of sensitized cells were prepared as follows: To a known volume of sheep's blood were added 10 units of hemolytic serum and several volumes of 0.14 N NaCl (0.85 per cent). After 1 hour's incubation at 37°C., the cells were sedimented by slow

centrifugation, washed once in 20 to 30 volumes of saline and resuspended to 10 times the original volume of blood, making a stock 10 per cent suspension of sensitized cells (as compared to whole blood).

In order to secure comparable results, a standard method must be used, in which all variables save that studied are kept constant.

Volume.—All tubes are made up to total volume of 2 cc.

Cells.—0.5 cc. of the 10 per cent stock suspension of sensitized cells.

Diluents.—Both the 0.14 N NaCl and 5 per cent glucose used for dilution were brought to pH 7.4.

Incubation.—1 hour at 37°C., followed by at least 2 hours in ice box, or 1 hour at room temperature.

Reading.—

0 = No observable hemolysis.
± = Doubtful.
1 = Distinct partial hemolysis.
2 = Approximately half of cells hemolyzed.
3 = Distinct residual opacity of non-hemolyzed cells.
4 = Complete hemolysis.

The hemolytic unit of complement is arbitrarily defined as the minimal quantity which will completely hemolyze 0.5 cc. of sensitized cells at pH 7.4 in 1/7 N NaCl under the above conditions of time and temperature.

(*b*) *Complement Fixation as Described in a Preceding Paper* (5).—As in the case of the hemolysis tests a uniform volume pH, tonicity, and time of incubation must be rigidly adhered to for comparable results.

(*c*) *Electrolytes.*—

NaCl	Na_2 HPO_4	$CaCl_2$
KNO_3	Na_2 SO_4	$BaCl_2$
$LiNo_3$	Na_2 Citrate[3]	$MgCl_2$
KCNS	Na_2 Tartrate	$MgSO_4$
LiCL	Na_2 Succinate	
	K_2 Oxalate[3]	

[3] Neither K_2 oxalate nor Na_2 citrate could be used. The former, in concentration > 0.2 N, causes a slow hemolysis accompanied by a change in tint, in the absence of complement; the anti-complementary action of the latter is well known, and, as yet, unexplained.

All the salts were prepared in 1 N solution and brought to pH 7.4 by addition of HCl (NaOH) before making the final dilution.

(*d*) *Hydrogen Ion Concentration.*—This was determined colorimetrically, and checked occasionally by hydrogen electrode. The figures as given are accurate only to 0.1 pH. The shift caused by the addition of sensitized cells, significant even in strongly buffered solutions, was determined by centrifuging out the cells after sufficient time had been allowed for equilibrium to become established.

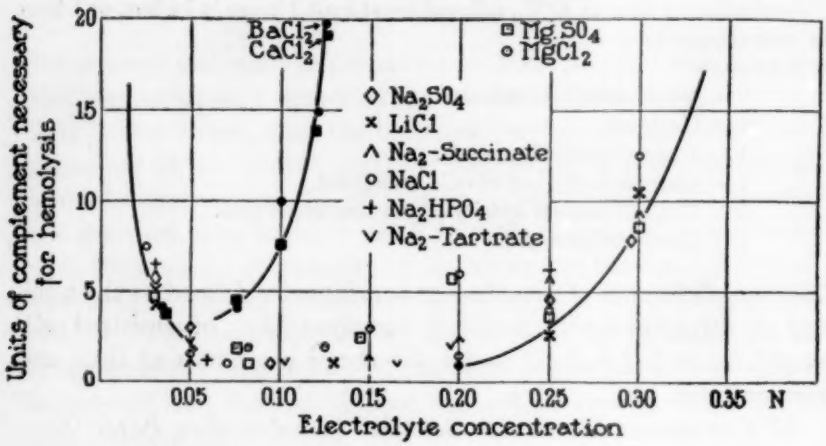

FIG. 4. Effect of electrolyte concentration upon hemolysis by complement.

2. The Effect of Electrolytes.

To a series of tubes containing increasing quantities of electrolytes were added 0.5 cc. of sensitized cells, washed and suspended in 5 per cent glucose, and 5 per cent glucose to a total volume of 2 cc. The minimal quantity of complement necessary for complete hemolysis was then determined for each concentration.[4]

[4] These experiments were carried out before there was available a rapid method of complement titration. That used here is cumbersome and relatively less precise than either the method of Brooks (2) or that used in experiments already described (4).

Table I gives the data for NaCl. To simplify comparison, the results obtained with the other salts are combined in Fig. 4, in which abscissae represent electrolyte concentration and ordinates the number of complement units necessary for hemolysis.

By varying the electrolyte concentration, three distinct effects are produced upon the hemolytic activity of complement. Below a concentration of 0.025 to 0.03 N, even 20 units of complement fail to cause hemolysis, provided of course, the electrolyte in complement is

TABLE I.

Influence of [NaCl] upon Hemolysis of Sensitized Cells by Complement.

		ISOTONIC								HYPERTONIC			
NaCl N/1, cc.			0.025	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5	0.6	0.8
H_2O, cc.			0.15	0.3	0.6	0.9	1.2	1.25	1.2	1.1	1.0	0.9	0 7
Glucose 5 per cent, cc.		1.5	1.3	1.15	0.8	0.45	0.1	0	0	0	0	0	0
Sensitized cells in glucose 5 per cent.		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Final [NaCl] N.			0.012	0.025	0.05	0.07	0.1	0.12	0.15	0.2	0.25	0.3	0.4
Complement													
cc.	units												
0.015	¾	0	0	±	3	4−	4−	4−	4−	2	0	0	0
0.02	1	0	0	1	4−	4	4	4	4	3	1	0	0
0.025	1¼	0	0	1	4					4−	1	0	0
0.03	1½	0	1	2	4					4	2	0	0
0.04	2	1	2	2							3	1	0
0.05	2½	2	0?	2							3	2	0
0.08	4	2	2	3							4	2	0
0.1	5	2	2	4−								4−	1
0.2	10	2	4−	4								4−	2
0.4	20	2	4	4								4	2
Minimal units complement necessary for hemolysis		>20	10–20	5–10	1–2	1	1	1	1	1½	4	10–20	>20

included in the calculation of the final salt concentration. There follows a zone of optimal concentration, whose upper limit for monovalent salts seems to be serum tonicity. Beyond this, increasing electrolyte concentration necessitates more and more complement, until, in 0.4 N NaCl the inhibition is absolute and even 20 units of complement fail to cause hemolysis.[5]

[5] The puzzling observation of Muir and Browning (15) that hypertonic salt allows complement to pass through a Berkefeld filter, by which it is usually re-

Neither of these two zones of inhibition is due to a destruction of complement. From hypertonic solutions, active complement may be recovered quantitatively by making the proper dilution (Table II). It is true that in hypotonic solution there is an irreversible inactivation of complement function. But in the presence of sensitized red cells this destruction is only partial. Here also, sufficient complement

TABLE II.

Survival of Complement in Hypertonic Solution.

To 0.5 cc. complement are added 0.5 cc. of N/1 electrolyte. After 1 hour at 37°C., the minimal hemolytic quantity of each mixture is determined, using sensitized cells in glucose to avoid hypertonicity.

Mixture of complement and electrolyte	NaCl	LiCl	$BaCl_2$	Control (NaCl N/7)
cc.				
0.02	2	3	2	3
0.03	4–	4–	3	4–
0.04	4	4	4	4
0.05	4	4	4	4

remains to completely hemolyze the cells, were it not for an as yet unexplained inhibiting factor; for when the proper tonicity is restored, hemolysis occurs (Table III).

But the most striking feature of the curves plotted in Fig. 4 is the influence of cation valence upon the second zone of inhibition, becoming even more manifest if we prevent the partial hemolysis caused by Ca and Mg salts (Fig. 5). The valence of the anion seems to play no rôle—but with increasing cation valence there is an astonishingly greater inhibitory action. The zone of optimal concentration is narrowed, and the succeeding inhibition much more pronounced. At

tained, is explained by these findings. Complement fails to pass the filter, not because of its molecular dimensions, but because it is adsorbed by the finely particulate material of which the filter is made. Hypertonic salt inhibiting such adsorption, the complement passes.

Similarly, the fact that after repeated passages of fresh serum, a filter finally becomes permeable to complement, is due to the saturation of the filter adsorbent with the complement adsorbate.

TABLE III.

Inhibition of Hemolysis in Hypotonic Solution not Entirely Due to Destruction of Complement.

Complement		Inhibition of hemolysis		Destruction in absence of cells	Control
		(a)	(b)		
		Cells 0.5 cc. Glucose 1.3 cc. Complement 37° 1 hour	NaCl N/1 −0.2 cc. added after incubation	Glucose 1.3 cc. Complement After 1 hour at 37° add Cells 0.5 cc. NaCl N/1 0.2 cc.	Glucose 1.3 cc. Cells 0.5 cc. NaCl N/1 0.2 cc.
cc.	*units*				
0.015	¾	0	2	0	3
0.02	1	0	3	1	4
0.04	2	0	4	3	4
0.05	2½	0	4	4−	4

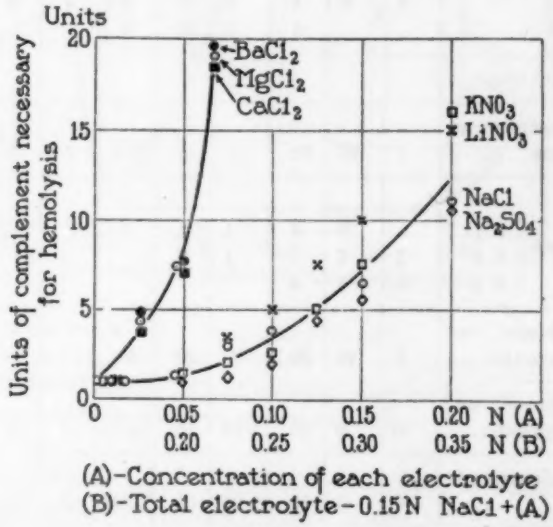

FIG. 5. Effect of electrolytes upon hemolysis (non-specific hemolysis by salts prevented in hypertonic solution).

To 0.5 cc. of sensitized cells in 0.15 N NaCl are added *X* cc. of N/1 electrolytes, each containing, in addition to the individual salt, 0.85 per cent NaCl (0.15 N). 0.15 N NaCl is added to a total volume of 2 cc., and the minimal hemolytic quantity of complement determined at each concentration.

The hemolysis caused by the salts themselves is almost completely inhibited in such hypertonic solutions.

TABLE IV.

Effect of Electrolyte Concentration upon Fixation of Complement by an Immune Globulin Suspension.

Complement is incubated with globulin at varying concentrations of NaCl. After 1 hour at 37°, to *Y* cc. of each tube (figures in left hand column), are added 0.5 cc. sensitized cells, and NaCl N/7 to a total volume of 2 cc. The figures in the body of the table represent degrees of hemolysis.

Globulin suspension, *cc.*		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Complement, *cc.*	0.2	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
NaCl N/7, *cc.*		0.25	0.25	0.5	1.5	1.45	1.4	1.35	1.3	1.25
NaCl N/1, *cc.*						0.05	0.1	0.15	0.2	0.25
Glucose 5 per cent, *cc.*	1.7	1.5	1.25	1.0						
Final NaCl normality	0.014	0.035	0.055	0.07	0.14	0.16	0.18	0.21	0.23	0.25
cc.										
0.1	±	3	1	0	0	±	1	2	3	4−
0.2	3	4	4	0	2	3	3	3	4−	4
0.3	4	4	4	0	3	4−	4	4	4	4
0.5			4	2	4	4				
0.6				2						
1.0				3						
Total units complement remaining free	7	10	10	<2	4	4–6	7	7	7–10	10–20
cc.										
Control without globulin 0.1		3	4	4	4	4				
Control without globulin 0.2	3	4	4	4	4					
Control without globulin 0.3	4	4	4							
Units complement remaining in control	7	10	20	20	20	20				
Units complement adsorbed by globulin	0	0	10	>18	16	14–16	13	13	10–13	0–10
Per cent complement adsorbed	0	0	50	>90	80	70–80	65	65	50–65	0–50
Amount of complement from which one unit would be adsorbed	>20	>20	2	1+	1+	1+	1+	1+	1–2	2–20

0.07 N $BaCl_2$ it required 20 times as much complement to cause hemolysis as it does at 0.07 N NaCl. The limiting concentration for hemolysis where even 20 units fail to have any effect is 0.35 M KNO_3, but only 0.07 M $CaCl_2$.

As compared with such a pronounced valence effect, the differences between individual ions of the same valence are negligible, and do not justify their arrangement into an ionic series.

If we now carry out a similar series of experiments to determine the effects of electrolytes upon complement fixation, we obtain a series of

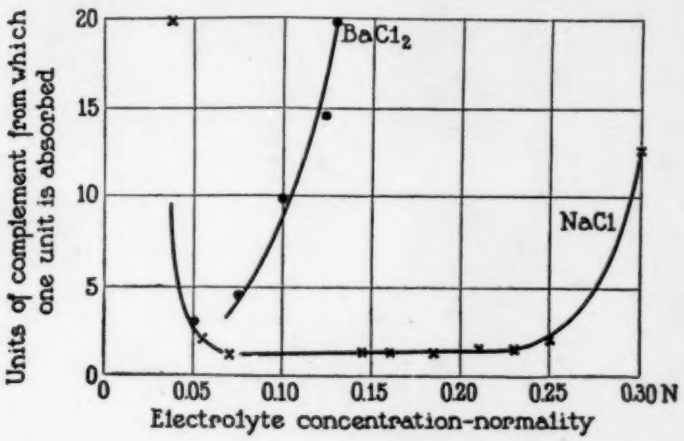

FIG. 6. Effect of electrolytes upon complement fixation.

With the more accurate velocity method of complement titration, one obtains a somewhat more sharply defined optimum zone for fixation. The limiting electrolyte concentrations, however, remain the same.

curves which are, within the limits of experimental error, exact duplicates of the curves for hemolysis. There is the same inhibition in hypotonic solution, the same optimum zone, and the same succeeding inhibition in hypertonic solution, again determined by the cation (Table IV and Fig. 6).

Knowing as we do that sensitized cells adsorb complement, the correlation between adsorption and hemolysis proves that the inhibition of hemolysis with changing tonicity is due to inhibited adsorption —that adsorption is an essential preliminary to hemolysis.

3. The Effect of Hydrogen Ion Concentration.

The effect of changing pH upon hemolysis is summarized in Fig. 7. The irreversible destruction of complement in alkaline reaction is seen to begin at pH 8.5. Its causes are unknown, and need not concern us here. In the acid range, the hemolytic titer of complement begins to decrease at pH 5.9 as compared with 5.3 under the conditions used by Brooks. But it should be noted that sensitized cells in some

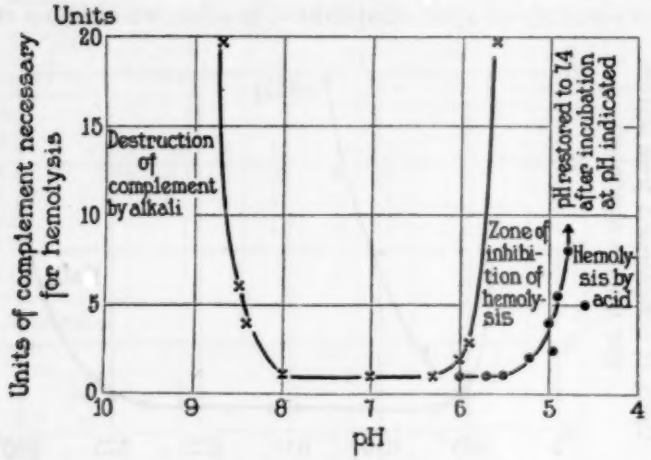

FIG. 7. Effect of hydrogen ion concentration upon hemolysis.

Complement is brought to the desired pH by the addition of NaOH N/7 (HCl). To *X* cc. of this mixture are added 0.5 cc. of sensitized cells and NaCl N/7 to a total volume of 2 cc. A significant change in reaction occurs when cells are added, and this must be determined for each quantity of complement used.

way prevent the destruction of complement between pH 5.9 to 5.0. When complement is brought to this pH and sensitized cells added at once, hemolysis does not occur, but not because the complement is destroyed, as it would have been in the absence of cells. When the optimum pH is subsequently restored by the addition of NaOH, hemolysis occurs, just as Michaelis and Skwirsky showed. The mechanism of this protective action is intimately related to the problem of midpiece and endpiece. Of interest in the present discussion

TABLE V.

Effect of pH upon Complement Fixation by Immune Globulin Suspension.

20 units of complement (0.4 cc.) are brought to the desired pH by acetate buffer, as indicated in the table, and incubated at 37°C. for 1 hour. To *Y* cc. of each tube (figures in left hand column), are then added 1), the neutralizing quantity of NaOH, 2), sensitized cells, and 3), NaCl N/7 to make 2 cc. The figures in body of table indicate degrees of hemolysis. The units remaining after incubation obviously $= \frac{2}{\text{minimal hemolytic quantity}^*}$

HAc N/7 (in NaCl), cc.		0.05	0.07	0.1	0.15	0.22	0.3
NaAc N/7, cc.		0.3	0.3	0.3	0.3	0.3	0.3
Complement, cc.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Globulin in suspension, cc.	0.1	0.1	0.1	0.1	0.1	0.1	0.1
NaCl N/7, cc.	1.5	1.15	1.1	1.1	1.05	1.0	0.9
Final pH	7.4	6.5	6.2	6.0	5.7	5.4	5.1
cc.							
0.05	0	0	0	0	3	2	1
0.1	0	0	0	1	4	4	4
0.2	0	0	2	4	4		
0.5	0	0	2	4			
1.0	0	0	3				
Total units remaining free	0	0	<2	10	20	20	20
cc.							
Control without globulin 0.05	3	3	3	3	3	2	1
Control without globulin 0.1	4	4	4	4	4	4	3
Control without globulin 0.2	4	4	4	4	4	4	4
Units remaining in control	20	20	20	20	20	20	10*
Per cent complement adsorbed by globulin suspension	100	100	>90	50	0	0	0
Amount of complement from which one unit would be adsorbed	1	1	1+	2	>20	>20	>20

* Beginning destruction of complement by acid reaction.

is the fact that there is a true zone of inhibition at pH 6.0 to 5.0, in which active complement fails to produce hemolysis.

Here also, the puzzling effects upon hemolysis are completely explained by the corresponding changes in complement adsorption by a suspension of globulin particles (and of red cells).

Table V is the protocol illustrating the method used to arrive at the data charted in Fig. 8. The inhibition of complement fixation at pH 5.9 quantitatively parallels and explains the inhibition of hemolysis.

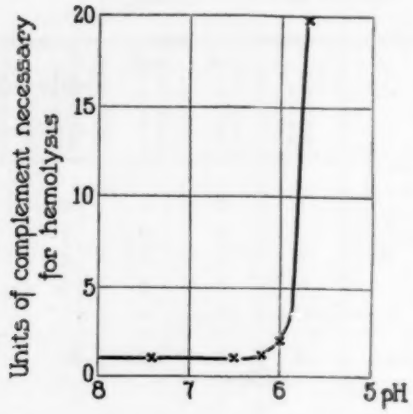

FIG. 8. Effect of pH upon complement fixation by a suspension of denatured immune globulins.

SUMMARY AND DISCUSSION.

1. Sensitization confers upon the red cell the property of adsorbing complement from solution. The submicroscopic film of immune serum protein deposited upon the cell surface during sensitization, and completely analogous to the precipitate formed in a soluble antigen-antibody reaction (*e.g.*, sheep serum vs. rabbit anti-sheep serum) acts as absorbent, the degree of sensitization (size of the film) determining the amount of complement "fixed" (adsorbed).

2. This adsorption of complement by the sensitized cell is an essential preliminary to hemolysis, and when inhibited, even large quantities of demonstrably active complement have no hemolytic action.

The marked influence of electrolytes and of the hydrogen ion concentration upon hemolysis is due primarily to corresponding effects upon the fixation of complement by the sensitized cell. In the case of salts with monovalent cations, complement fixation (and hemolysis) is completely inhibited at any concentration < 0.02 M or > 0.35 M. Electrolytes with bivalent cations are much more inhibitory, and in low as concentration 0.07 M completely prevent fixation (and hemolysis).

The optimal reaction for complement fixation (and hemolysis) is pH 6.5 to 8.0. In slightly more acid range both are *inhibited*. But at a reaction pH 5.3, and in the alkaline range, there is an irreversible inactivation of complement, complete at pH 4.8 and 8.8 respectively.

It is perhaps more than a coincidence that complement fixation, and therefore, hemolysis, are prevented by just those factors which suppress the ionization of serum proteins, and lead to an increased aggregation state. Between a suspension of macroscopically visible particles of euglobulin in distilled water, and a solution is physiological saline, there is certainly a gradual transition, manifested at low electrolyte concentrations by the opacity of the solution. At pH 7.4, globulin would ionize as a Na-salt, an ionization inhibited as the isoelectric point (5.3) is approached, with a coincident greater tendency of the globulin to separate from solution. And the cataphoretic velocity of particles of globulin, as well as all the other properties which are a function of its ionization (viscosity, osmotic pressure, etc.), are suppressed by electrolytes, the degree of suppression being determined by the concentration and valence of the cation (on the alkaline side of the isoelectric point). The analogy with complement fixation is too complete to be dismissed as fortuitous.

3. The fact that the degree of complement "fixation" increases with the degree of sensitization explains one of the most puzzling phenomena in hemolysis,—that immune serum and complement are, to a certain extent, interchangeable, a decrease in either factor being compensated by an increase in the other (8), (20), (22). The explanation is evident from Figs. 1, 2, and 3. The exact quantitative relationships involved will be developed in a later paper. With increasing sensitization there is an enormously more complete and more rapid fixation of complement, and correspondingly more rapid hemolysis, exactly

the effect produced by increasing the quantity of complement instead of amboceptor (Fig. 3). All other variables being constant, the velocity of hemolysis is determined by the amount of complement adsorbed. With more amboceptor, a greater proportion is "fixed" by the cell; with more complement, a smaller proportion, but a larger absolute amount. The result is the same: more complement adsorbed, and a corresponding acceleration of hemolysis.

If this mobilization of complement is the sole function of immune-serum (and there is as yet no reason to assume any other), then the accepted terminology, in which amboceptor, immune body, and hemolysin are used synonymously, is erroneous. The immune body would function only as an "amboceptor," mobilizing the effective hemolysin, complement, upon the surface of the cell.

Nothing has been said of the multiple components into which complement may be split. *A priori*, it would be expected that the adsorption demonstrated is of the so called midpiece fraction.

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THE PENETRATION OF LUMINOUS BACTERIA BY THE AMMONIUM SALTS OF THE LOWER FATTY ACIDS.

PART I. GENERAL OUTLINE OF THE PROBLEM, AND THE EFFECTS OF STRONG ACIDS AND ALKALIES.

BY SAMUEL E. HILL.

(From the Physiological Laboratory, Princeton University, Princeton.)*

(Accepted for publication, March 26, 1929.)

In the experiments of Jacobs[1] on hemolysis by the ammonium salts of the lower fatty acids, hemolysis is used as a measure of penetration. It is concluded that only the molecules of ammonia and of fatty acid, formed by hydrolysis of the salt, are effective in penetrating the cell membrane. These reunite within the cell to form the salt, cytolysis resulting from the increased osmotic pressure.

It was thought that some of these studies might be repeated, using luminous bacteria as the experimental material. The problem as originally conceived was a study of the rates of cytolysis of luminous bacteria by the ammonium salts of formic, acetic, propionic, butyric, and valeric acids, using solutions of all pH values at which measurements of the time of cytolysis could be made. Cytolysis would be detected by the disappearance of the light and would indicate penetration. As the work progressed it became apparent that disappearance of the light did not always indicate cytolysis but under the conditions of the experiments did indicate penetration, and numerous modifications of the original procedure were suggested. Before attempting

* The experiments described herein were carried out in the physiological laboratory of Princeton University and in the laboratory of the United States Bureau of Fisheries. I wish to acknowledge my indebtedness to Mr. Henry O'Malley, Commissioner of Fisheries, and to Mr. Elmer Higgins, Director of the Bureau of Fisheries Laboratory at Woods Hole, for placing the facilities of the laboratory at my disposal. I am also deeply indebted to Prof. E. Newton Harvey of Princeton University, under whose direction this work was done, for constant advice and guidance.

[1] Jacobs, M. H., Harvey lectures, 1927, xxii, 146.

analysis of the data obtained with the ammonium salts it was therefore necessary to answer a number of questions bearing on these points, as follows:

Are luminous bacteria cytolyzed as erythrocytes are by water, hypotonic non-penetrating salt solutions, and by penetrating solutions of all concentrations? Is disappearance of the light coincident with cytolysis? May disappearance of the light be used as a criterion of cell penetration? May the presence of the ammonium salts within the cell be responsible for the extinction of the light, independently of osmotic effects? Are the bacteria injured by the activity of hydrogen and hydroxyl ions in the range of pH values covered by the ammonium salts? Is the point of injury the same, whether hydrogen or hydroxyl ion activity is exerted on the outer surface of the cell membrane or on the protoplasm itself? May salt injury and acid and base injury be separated from the osmotic effects of the ammonium salts?

These questions will be considered under subsequent headings.

I.

Experimental Methods.

The organism studied was *Bacillus Fischeri* (Beijerinck, Migula), a marine form isolated from herring by C. S. Shoup at Woods Hole during the summer of 1927 and since maintained in pure culture in my laboratory. The bacteria were grown in Petri dishes, using the calcium carbonate buffered media described in a previous paper.[2] The cultures were used when approximately 24 hours old, the bacteria being brushed from the surface of the medium and collected in M/2 NaCl.

A single drop of bacteria suspended in M/2 NaCl was added to 10 cc. of solution, the tube vigorously shaken, and watched until the light disappeared. In some early experiments the attempt was made to use a standard low intensity of light as the end-point of the reaction. The results thus obtained were so erratic and unreliable that these experiments were discarded and total disappearance of the light taken as the end-point. The eye is sensitive to light of so low an intensity that measurements are difficult, and Reeves[3] has shown that this threshold varies from day to day. In order to minimize errors due to this variability as well as to variability in the bacteria, experiments were always run in multiple. No measure-

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[2] Hill, S. E., *Biol. Bull.*, 1928, lv, 143.

[3] Reeves, P., *Abridged Scient. Pub. Research Lab. Eastman Kodak Co.*, 1917–18, iii, 13.

ments are recorded except those made after at least 1 hour spent in light of low intensity, and at least 15 minutes in total darkness. The use of a stop watch under such conditions is obviously impossible. The timing unit was therefore a metronome adjusted to beat seconds. The experiments were run in a definite order and the results recorded on a "Sunstrand" adding machine, which possesses a type of keyboard that can be operated in the dark without error. The records were transcribed to sheets at the close of the experiment.

The pH values of the solutions were measured with either the quinhydrone or the glass electrode, and in a few cases calculated values were used, as for NaOH solutions above pH 9.0. In every case, the method of determining pH is given.

It was early found that a single suspension of bacteria could not be kept in good condition for the 4 or 5 hours necessary to run the longer experiments. In the experiments which were run in multiple, a separate culture of bacteria was used for each of the 8 or 9 parts. The suspension was aerated with a stream of air. In each case the first experiment was run from the acid to the alkaline end of the series, the second in reverse order, etc., in order to equalize any differences due to ageing of the suspension, but no definite differences were found. In all cases the average time of disappearance of the light in 8 or 9 experiments was plotted on the vertical axis and the concentration of free acid or alkali, or pH of the solutions on the horizontal axis.

II.

Applicability of Method.

Luminous bacteria are cytolyzed by water, hypotonic non-penetrating solutions, and by penetrating solutions of all concentrations. This cytolysis differs from that of erythrocytes because of the difference in shape of the cell, and because the bacillus possesses a rigid cell wall which prevents swelling. The mammalian erythrocyte, because of its peculiar shape, can increase considerably in volume without an increase in surface.[1] The surface membrane can then stretch to some extent before any great resistance is offered to osmotic swelling. With the bacillus, great mechanical resistance is at once offered to osmotic entrance of water, and the cell bursts without increasing in size. The evidence on which these statements are made is presented below.

If luminous bacteria are placed in diluted sea water, there is little change in intensity of the light until the sea water decreases to 20 per cent. Solutions from 20 to 6 per cent sea water form a series of decreasing brilliance, and after a lapse of 5 minutes no light is observed in solutions more dilute than 6 per cent sea water. Viewed in day-

analysis of the data obtained with the ammonium salts it was therefore necessary to answer a number of questions bearing on these points, as follows:

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light, the solutions were decreasingly turbid with decreasing sea water concentration, a marked difference being observed between the 100 cent and 6 per cent solutions. On vigorous shaking, foam developed in all of the solutions, but persisted only in those of sea water concentration below 6 per cent. This indicated the bursting of the cell membrane and the liberation in the solutions of foam-producing substances such as the cell proteins. Examined by transmitted light, the bacteria in the solutions of less than 6 per cent sea water were almost impossible to find but were easily demonstrated in the dark field. In solutions of above 6 per cent they were demonstrable also by transmitted light. Mrs. E. B. Harvey kindly measured a number of bacteria for me, finding only a slight increase in size in 20 per cent sea water.

In another experiment carefully measured quantities of bacterial suspension were added to a series of sea water dilutions as before and counts of the bacteria made in the dark field. No significant differences in number were observed even between 2 per cent and 100 per cent sea water. As before, when the solutions were examined by transmitted light it was difficult or impossible to demonstrate the bacteria in the more dilute samples.

The difficulty of demonstration of cytolyzed bacteria by transmitted light is thought to be due to the filling of the cell capsule with water, so that light passing through is not appreciably deflected from its course. With dark field illumination the surface only is concerned and the effect is therefore the same whether we are dealing with a normal cell or a cytolyzed one.

In distilled water or tap water the light disappeared in about 15 seconds. The presence of low concentrations of NaCl (up to 0.0156 M) delayed the disappearance of the light for a few seconds and in solutions of 0.0625 M and upward the light lasted for over 1 hour although greatly dimmed in 0.0625 M NaCl. The persistence of the light, undimmed, through a considerable range of NaCl dilution and its great dimming shown progressively through several dilutions, followed by sudden disappearance in slightly more dilute solutions, indicate the rupturing of the cell membrane at a critical osmotic pressure, and the presence of different degrees of resistance in the bacterial population. The least resistant were cytolyzed by 0.0625 M NaCl and the most

resistant by 0.0156 M NaCl. These differences might be individual variation or a combination of individual variation with age groups in the population. The results of microscopic examination of the NaCl dilutions were in every way similar to those of the sea water dilutions.

Sucrose was almost as efficient as sea water or NaCl in preserving the luminescence, as the light disappeared in solutions of 0.0625 M and below, and exhibited a series of increasing brilliance as the sugar concentration rose. This indicated that the failure of light in diluted sea water was an osmotic phenomenon due to bursting and was not

TABLE I.

Time Required for Complete Disappearance of Light of Luminous Bacteria in Hypotonic Solutions.

No examination was made after 6 hours.

Per cent of sea water		Concentration of NaCl		Concentration of sucrose	
100.0	+	0.5 M	+	1.0 M	+
50.0	+	0.25	+	0.5	+
25.0	+	0.125	+	0.25	6 hrs.
12.5	6 hrs.	0.0625	6 hrs.	0.125	1 hr.
6.0	5 min.	0.0312	5 min.	0.0625	5 min.
3.0	1 "	0.0156	1 "	0.0312	1 "

due to the absence of the customary salts as such. This had been previously observed by Harvey[4] for an unindentified species of luminous bacteria.

In solutions of ethyl alcohol of all concentrations from molar downward the light disappeared in a few seconds as in pure water, while in presence of sea water or 0.5 M NaCl similar concentrations of alcohol were without effect. This shows that the effect of the alcohol in pure water was caused by its easy penetration, and was not due in any way to narcosis or to an attack on the cell membrane.

In solutions of glycerine from molar downward the time of disappearance of the light was scarcely distinguishable from that in pure water, being perhaps 20 per cent longer. In presence of 0.5 M NaCl

[4] Harvey, E. Newton, *Biol. Bull.*, 1915, xxix, 308.

these concentrations of glycerine were without effect on the luminescence, showing that glycerine also penetrates these cells with great readiness.

Urea did not penetrate as readily as alcohol and glycerine, the disappearance of the light occurring in M urea in about three times the time required in pure water (Table II). As before, addition of 0.5 M NaCl prevented the disappearance of the light.

It will therefore be seen that with hypotonic or with freely penetrating non-injurious substances disappearance of the light is undoubtedly coincident with cytolysis. With freely penetrating

TABLE II.

Time of Disappearance of Light in Indicated Concentrations of Urea.

[Urea]	In water	In 0.5 M NaCl
1.0 M.	41 sec.	+8 hrs.
0.8 "	38 "	+8 "
0.6 "	36 "	+8 "
0.4 "	32 "	+8 "
0.2 "	29 "	+8 "
0 "	15 "	+8 "

injurious substances the cell processes may be interfered with and disappearance of the light thus precede cytolysis, or cytolysis may not occur. Since the luminescence of bacteria is intracellular, as has been shown by Harvey[5] and others, any change in intensity of light must be due to changes within the cell. The disappearance of the light indicates cytolysis, anesthesia, the presence of some destructive substance within the cell, or cessation of function because of unfavorable environmental conditions, especially lack of oxygen.

The light may be reversibly extinguished in several ways, as follows: (a) Increase of temperature above a certain critical value, not yet determined for this species, but above 30°. This point has been determined by several investigators for other species.[6,7,8] (b)

[5] Harvey, E. Newton, The nature of animal light, Philadelphia, 1919.
[6] McKenney, R. E. B., *Proc. Biol. Soc. Washington*, 1902, xv, 213.
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Decrease of oxygen pressure to a value less than 0.0053 mm. Hg.[9] (c) Presence of anesthetics in sub-lethal concentrations.[6] (d) By concentrated non-penetrating salt solutions, as by 2.5 M NaCl, the disappearance of the light in this case being due doubtless to plasmolysis.

In all experiments described in this paper concentrated salt solutions have been avoided, presence of ample quantities of oxygen has been assured, and all experiments were conducted at 19 ± 1°C. The disappearance of the light under the conditions of the experiments must therefore be due to osmotic swelling of the cell or to the entrance of injurious substances. When isotonic solutions are tested the disappearance of the light may be used as a physiological criterion of cell penetration whether the failure of the light is due to osmotic swelling or to injury due to the presence of some foreign substance within the cell. The case of internal injury, distinct from osmosis, may be detected by the addition of 0.5 M NaCl to the solutions, thus preventing cytolysis. In any case, disappearance of the light is an indication of cell penetration.

With non-penetrating substances such as strong acids and bases entrance would probably follow destruction of the cell membrane. Disappearance of the light might follow injury to the membrane, and precede entrance of the destructive agent to the cell. Entrance after destruction of the cell membrane can hardly be regarded as penetration, but this case is easily detected by the behavior described in the next section.

III.

Effect of Strong Acids and Alkalies.

The experiments described below indicate that injury due to activity of hydrogen and hydroxyl ions does not take place within the range of pH values employed with the ammonium salts. Therefore any disappearance of luminescence caused by the ammonium salts must be due to osmotic swelling or to intracellular changes due to penetration of the salts, or of their hydrolytic products.

Using the non-penetrating HCl in 0.5 M NaCl, the luminescence persisted for a few seconds even in concentrations as high as 0.1 M

[9] Harvey, E. Newton, and Morrison, Thos. F., *J. Gen. Physiol.*, 1923, vi, 13.

and lasted for progressively longer periods of time as the acid mixture was diluted with 0.5 M NaCl. At pH 4.0 the light lasted for over 5 minutes, at pH 4.67 for 20 minutes, and at pH 5.46 for over 4 hours. The nature of the curve for the shorter times is shown in Fig. 1. Since none of the ammonium salts employed were more acid than pH 5.0 and since the times measured with the ammonium salts were in all instances 5 minutes or less, it is obvious that injury due to activity of hydrogen ions in the external solutions may be excluded from consideration. Similar results were obtained with NaOH, a non-penetrating base. With one series the pH values were calculated on the assumption that NaOH is completely dissociated, and in the other the glass electrode was used, corrections being applied from the measurements of pH values of ammonia in water and in 0.5 M NaCl solution. In these two series of experiments, the pH was determined after the experiment, so the glass electrode determinations are probably more accurate than the calculated values, since some carbon dioxide was absorbed from the air and some produced by the bacteria in the course of the experiment. At pH 9.0, the upper pH value for most of the ammonium salts employed, the bacteria were still luminous after 2 hours, and it was only above pH 10.0 that any immediate effects could be observed. Damage due to activity of hydroxyl ions in the external solution may also be disregarded.

An outstanding peculiarity of the behavior of luminous bacteria was noted in both HCl and NaOH, as compared with the free fatty acids and with ammonia. In 0.025 M solution of any fatty acid in 0.5 M NaCl the disappearance of the light was so rapid that the time could not be measured, while in 0.025 M HCl in 0.5 M NaCl the light persisted fully brilliant for approximately 6 seconds, then failed completely in less than 1 second. In 0.025 M ammonia in 0.5 M NaCl the light was immediately dimmed to a very low value and then lasted for some time before being finally extinguished. In 0.025 M NaOH in 0.5 M NaCl the light lasted at full value for about 4 seconds, then disappeared suddenly. This complete resistance for a certain period of time, followed by sudden complete failure, indicates that the protoplasm is protected from the strong non-penetrating HCl or NaOH until the membrane is destroyed, the destruction of the then unprotected protoplasm following almost immediately. The HCl or NaOH

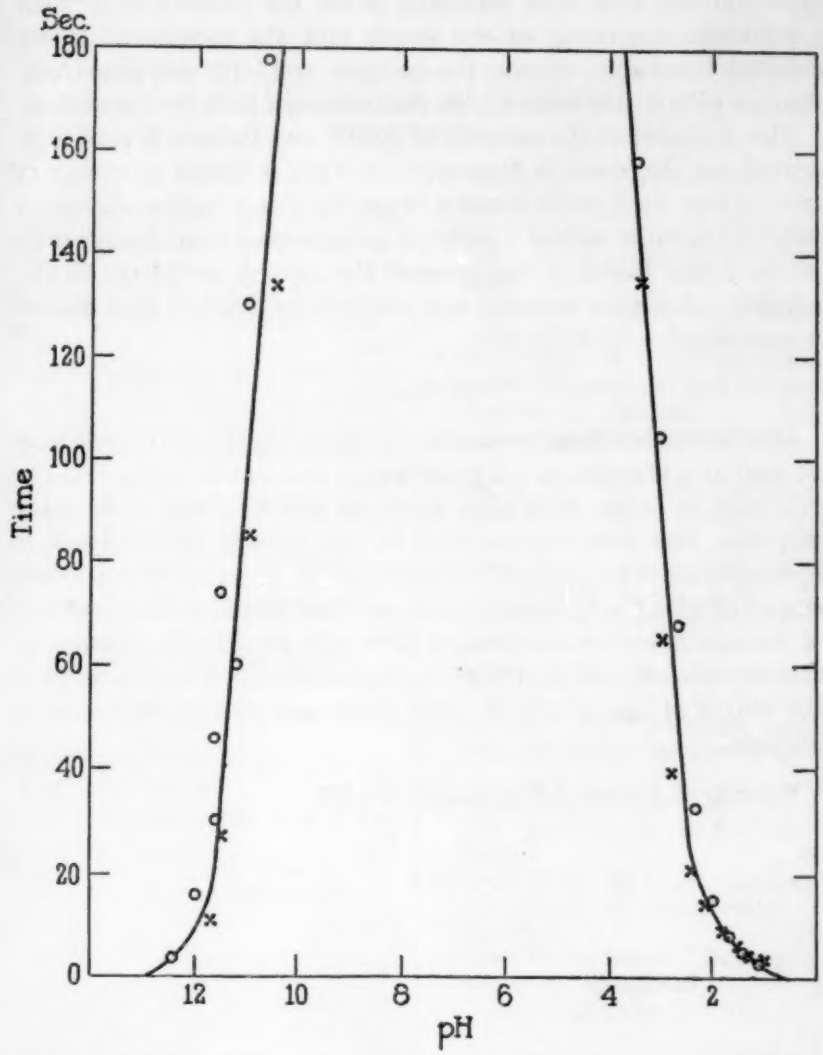

FIG. 1. Time of disappearance of light (in seconds) in solutions containing 0.5 M NaCl plus NaOH or HCl. Each circle is average of 9 experiments, each *X* of 10. For the circles, pH was determined with the quinhydrone electrode on the acid side, calculated on the alkaline side. For the *X*'s, pH was determined with the glass electrode, with appropriate corrections.

must combine with some substance in the cell membrane, forming a permeable substance, or else simply etch the membrane, before entrance is possible; whereas the ammonia and fatty acid pass freely through without the necessity for first making a path for themselves.

This is similar to the entrance of NaOH into Paramecia stained in neutral red, described by Harvey.[10] In 0.002 M NaOH no change in color is seen for 8 or 10 minutes, when there is a sudden change in color of the entire animal. In 0.001 M ammonia a color change from red to yellow begins at the moment the animals are placed in the solution. A similar behavior was observed for *Elodea* leaves stained in neutral red.

SUMMARY.

It is shown that disappearance of the light of luminous bacteria may be used as a criterion of cell penetration; that luminous bacteria are cytolyzed by water, hypotonic solutions, and by freely penetrating solutions; that luminous bacteria are not injured by hydrogen or hydroxyl ions in the external solutions within the range of pH values employed with the ammonium salts and that therefore disappearance of the light in isotonic solutions of these salts must be due to penetration of the solute; and that there is a characteristic difference between the effects of strong and of weak acids and alkalies on luminous bacteria.

[10] Harvey, E. Newton, *J. Exp. Zool.*, 1911, x, 507.

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